HORMONES

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ATHEROSCLEROSIS

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GREGORY PINCUS

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PREFACE

Recognizing the extent and importance of current investigations on the role of hormones in atherosclerosis and the need for critical and informed communication between the investigators in this general field the Endocrinology Study Section of the National Institutes of Health appointed a subcommittee to organize a conference that would bring together the various disciplines involved This committee consisted of Col Marshall E Groover Dr Samuel Gurin Dr Robert T Hill Dr Leo T Samuels Dr Jeremiah Stamler Dr Alfred E Wilhelmi, and Dr Gregory Pincus chairman Aided by a grant made to the Endocrinology Study Section by the National Heart Institute the committee organized a meeting which lasted from March 11th to March 14th 1958 The meeting was held at the Alpine Rose Lodge in Brighton Utah with the kind cooperation of Dr Leo Samuels and his colleagues at the University of Utah Medical School

The program of the meeting herein published involved the presentation of a series of papers and the recording of the discussions of these papers This program was designed to cover five major aspects of research pertinent to the problem of hormones and atherosclerosis First of all the problem of cholesterol metabolism is discussed in various aspects including the nature of cholesterol biosynthesis the hormonal influences thereon and certain considerations of cholesterol catabolism Second the role of hormones in lipogenesis and lipid transport particularly in relation to atheromatous lesions is discussed Third available data on the influence of various hormones on experimental atherosclerosis are reviewed Fourth the much discussed problem of the interrelationship between blood lipids and the endocrine state in animals and man is presented in detail Finally we have a series of papers on clinical biological interrelationships important to the consideration of endocrine influences on human atherosclerosis With this coverage of a wide range of investigations it is hoped that a thorough airing has been given to fundamental data and the concepts which have arisen from these data

Stated in the simplest possible terms there is abundant evidence that cholesterol biosynthesis transport degradation and excretion may come under hormonal influence Furthermore different endocrine systems may have different effects upon these processes Not all of the endocrine influences are clear cut and vividly definable Also the fundamental concept that the nature of cholesterol metabolism in the mammals affects in one way or another the phenomenon of atherosclerosis may be questioned either in detail or in extenso Nonetheless hor

monal influences on the basic processes are definitively indicated and a discussion of these influences is clearly worth while Again, it is elementary that one may establish atherosclerotic lesions along with accompanying blood phenomena in experimental animals In these conditions hormones may act both prophylactically and therapeutically How relevant the results with experimental animals are to human atherosclerosis is certainly a matter for discussion

Experimental data presented here as well as a host of observations in the literature demonstrate conclusively that the sex hormones thyroid hormone and adrenocortical hormones may definitively affect the level of circulating blood lipids in man Although a correlation appears to exist between these blood lipid levels and the degree of development of atherosclerosis a major problem is whether the hormonal effects on lipid levels are also effects on tissue atherosclerosis The possibility of a disengagement of the factors concerned with blood levels from those concerned with tissue lesion development certainly requires exploration

The cardinal question for therapy in atherosclerotic disease is the utility of hormones as therapeutic agents This is certainly discussed in detail in the latter part of this book However equally important is the problem of the role of hormones in the etiology of human disease Attempts at rational therapy thus far have perhaps ignored the pro atherosclerotic effect of certain endocrine states

It is the hope of the committee that this presentation of the discussion of the foregoing problems and related matters will be welcomed for purposes of orientation in this complex field In addition we feel that a stimulation to further critical inquiry may be one of the fruits of the efforts here incorporated We believe that you will find in these pages clear evidence of the devoted pursuit of investigation on the part of the participants in the symposium To these participants the committee is extremely grateful

GREGORY PINCUS

Shrewsbury Massachusett
November 1958

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CHAPTER 1

Biosynthesis of Cholesterol

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If the problem of atherosclerosis were merely a matter of under standing the mechanism of cholesterol biogenesis one would today indeed be entitled to optimism Lately there has been considerable progress in the understanding of this biosynthetic process and judging from the rate of advance a knowledge of all the essential facts about cholesterol biogenesis now seems imminent It will be useful to begin by pointing out the four major phases of cholesterol biogenesis which can be experimentally separated

The first of these is the branching reaction which involves the coupling of three molecules of acetyl CoA (acetyl coenzyme A) to β hydroxy β methylglutaryl CoA and its reduction to mevalonic acid (MVA) Over all these reactions involve the elimination of two molecules of water (or CoA) and the reduction of a carboxyl function In the second stage MVA molecules are polymerized to the hydrocarbon squalene which is the immediate acyclic precursor of the steroids This over all transforma tion is also reductive and entails the uptake of two hydrogen atoms and the elimination of twelve molecules of water and of six molecules of carbon dioxide Stages 1 and 2 which cover the reactions from acetic acid to squalene are thus anaerobic in nature and it has indeed been shown by Bucher (3) that in isolated liver kept under strictly anaerobic conditions the transformations of acetic acid stop at the squalene stage All subsequent reactions are intimately associated with the oxidative metabolism of the tissues because the various oxygen atoms which enter in the course of cholesterol synthesis have their origin in molecular oxygen This is true for the cyclization of squalene which is oxidative (24) and for the oxidative removal of the three branched methyl groups of lanosterol (15) One may estimate that the biogenesis of cholesterol comprises altogether 20–30 separate enzymatic steps (2) only a few of which can now be studied at the enzymatic level It is perhaps not sur prising that the early steps which are concerned with transformations of soluble intermediates have yielded more readily to enzymatic analysis than the conversions beyond squalene

The transformation of acetyl CoA to acetoacetyl CoA initiates the synthesis of both the sterols and of the higher fatty acids but thereafter the two pathways diverge Reduction to β hydroxybutyric acid directs

the 4 carbon precursors towards the higher fatty acids while the coupling with a third molecule of acetyl CoA affords the branched chain building stones for terpene and steroid biogenesis (18) This separation of pathways applies not only to the molecular changes but also to the intracellular distribution of the enzymes Beta hydroxybutyric acid formation takes place in the soluble part of the cytoplasm (11) whereas the condensation to hydroxymethylglutaryl CoA is catalyzed by microsomal enzymes (18) Recently Rudney and his collaborators (7) have succeeded in closing the gap between hydroxymethylglutaryl CoA and MVA by demonstrating the enzymatic reduction of the thioester portion of the dicarboxylic acid The reductive steps of stage 1 which I mentioned earlier thus refer to the conversion of a CoA ester first to the aldehyde stage and then to the stage of the primary alcohol

Mevalonic acid which was first isolated as the acetate replacing growth factor for *Lactobacillus casei* (27) is now firmly established as an effective and presumably obligatory terpene and sterol precursor (22) Comparing the structure of mevalonic acid with the structural subunits of squalene one is tempted to look upon mevalonic acid as an incipient isoprene Elimination of two molecules of water and removal of the carboxyl group will afford the substituted butadiene without change in oxidation state and this is in fact what appears to happen enzymatically Working with soluble extracts of autolyzed yeast we have shown a requirement for ATP (adenosinetriphosphate) in the early stages of the MVA squalene conversion (1) Subsequently Tchen (23) was able to demonstrate that ATP interacts with MVA to form a monophosphate ester which is relatively stable to acid and alkali and therefore in all likelihood is the ester of a primary alcohol (MVA-5 phosphate) The further transformation of MVA monophosphate as determined either by the loss of the carboxyl group (C 1) or by squalene formation requires another reaction with ATP affording what appears to be a diphosphate of MVA (16) The two phosphorylation steps may be viewed as a means of facilitating the two dehydration steps by elimination of phosphate anion rather than of OH^- Our indications are that the immediately ensuing step is the removal of the carboxyl group possibly with the concurrent elimination of a phosphate residue While we lack direct evidence as to the structure of additional intermediates between MVA and squalene the outcome of various experiments with heavy hydrogen as a tracer has encouraged us to formulate the mechanism for squalene synthesis in considerable detail (17)

I have pointed to the fact that the terminal carbon atom of MVA (C 5) is reduced and it seemed important to us to ascertain whether this state of reduction persists throughout the synthesis of the poly

isoprenoid chain By preparing 2 C^{14}-5-di T MVA we were in a position to determine whether any hydrogen bound to C-5 is lost in the course of squalene formation Had the hydroxymethyl group been oxidized either to aldehyde or to carboxylic acid half or all of the carbon bound tritium should have been lost Analysis of the squalene derived from the doubly labeled MVA showed however that neither was the case (1) but that in fact all of the labeled hydrogen was retained This result was unexpected because in all known carbon carbon interactions at least one reactant carries a carbonyl function In squalene synthesis on the other hand we seem to be dealing with the novel case of carbon-chain formation by condensation of two active methylene groups Our results with doubly labeled MVA and independent experiments with D_2O have led us to conclude that isoprene presumably enzyme bound is formed by decarboxylation and phosphate elimination of MVA diphosphate According to our current views three molecules of isoprene or isoprene enzyme complex condense concertedly in a cation initiated process to form a sesquiterpenoid intermediate (C-15) This can stabilize either by proton elimination to farnesene by the uptake of OH^- to nerolidol or thirdly by isomerization and OH^- uptake to farnesol In this manner the structures of the naturally occurring acyclic sesquiterpenes can be readily rationalized

The hydrocarbon farnesene in turn provides an attractive structure for the reductive dimerization of two sesquiterpenoid units to squalene This condensation also is formulated as a concerted process a proton attacking one C-15 unit and a hydride ion the other (17) The mechanism which I have presented while speculative is the only one which accounts for two in our opinion significant results one of which is the retention of hydrogen at C-5 of MVA and the other the limited uptake of deuterium (3–4 atoms) by squalene synthesized in a D_2O medium We recognize the risk of attaching considerable weight to a few isotopic data yet we feel that our scheme even if proven wrong in detail is correct in principle and therefore useful as a working hypothesis If squalene synthesis should occur largely by concerted mechanisms many of the postulated intermediates are likely to elude isolation because of their transient nature The same dilemma exists for the tetracyclization reaction by which squalene is converted to lanosterol In their brilliant theoretical paper Ruzicka and his collaborators (19) formulate the transformation of squalene to lanosterol as a "non stop" reaction that is initiated by activated oxygen and leads to the steroid structure in a series of concerted electron displacements Our studies on the enzymatic cyclization of squalene to lanosterol have given firm experimental support to these postulates and we concur with the view that the tetracyclic

ring system is established without the stabilization of partially cyclized intermediates (25) The transformation of squalene to lanosterol entails a rearrangement of the carbon skeleton requiring an intramolecular shifting of either the methyl group at C 8 to the C 13 position or a shifting of two methyl groups to their adjacent position (from C 8 to C 14 and from C 14 to C 13) This important detail in the cyclization mechanism has now been settled in favor of the 1,2 methyl shift alternative (13) confirming the prediction by Eschenmoser *et al* (6) that only a 1,2 methyl shift is consonant with a fully concerted cyclization mechanism

Enzymatic studies on this interesting reaction, the system which we have referred to as the squalene oxidocyclase system have continued but have on the whole been disappointing We appear to be facing here a problem familiar to students of steroid biogenesis and steroid transformations namely the fact that these enzymes are intimately associated with the microsomal particles In our laboratory efforts to solubilize either the cyclizing system or the enzymes concerned with the demethylation of lanosterol to cholesterol have so far remained without success For the case of squalene cyclization by liver microsomes we have established a requirement for molecular oxygen—this being the source of the 3 hydroxy group—for a soluble enzyme which has been fractionated to some extent and may be a triphosphopyridine nucleotide (TPNH) oxidase and finally for a heat stable but so far elusive cofactor (4) Here as in other cases of enzymatic oxygenation the mechanism of oxygen activation remains obscure It is worth noting that in animal tissues the squalene molecule cyclizes exclusively to lanosterol i e in an asymmetric manner In plants the same acyclic precursor undergoes cyclizations in much greater variety viz., to pentacyclic triterpenes symmetrical tetracyclic products and to sterols as well

Lanosterol is a short lived intermediate and rapidly undergoes oxidative demethylation to cholestane derivatives at least in liver That the removal of the methyl groups at carbon atoms 4 and 14 is oxidative follows from the fact that these substituents are attached to quaternary carbon atoms Therefore structural considerations alone require an initial attack by oxygen In line with this contention we find that lanosterol is metabolically inert under anaerobic conditions and furthermore that there is stepwise methyl group oxidation by way of hydroxymethyl compounds aldehydes and carboxylic acid with eventual loss of CO_2 to the corresponding nor compounds (15) Last year we isolated and described an intermediate in the lanosterol cholesterol conversion to which the partial structure of a 14 norlanostadienol was assigned (8) By synthesizing the appropriate reference compounds we have now been able to identify this C 29 sterol as $\Delta^{8,24}$-4 4 dimethyl

cholestadienol (9). Thus the double bonds remain in the 8-9 position during the first demethylation step. The elimination of the two methyl groups remaining at C-4 also involves successive oxidations, i.e., first an attack upon one methyl group leading upon its removal to 4-monomethyl cholestane derivatives, observations in line with recent reports on the occurrence of 4α-methyl sterols in various natural sources (5, 14, 26). Studying the demethylation of lanosterol, we noted that some of the intermediates had the properties of ketones. We therefore prepared various sterols labeled with T (tritium) in the 3α position in order to localize the stage at which the 3-hydroxy group is oxidized. On enzymatic conversion of 3α-T-lanosterol or of 3α-T-14-norlanosterol to cholesterol the tritium was quantitatively lost. On the other hand, labeled hydrogen was fully retained during the conversion of 3α-T-zymosterol to cholesterol, and it therefore follows that the 3-hydroxy → 3-keto transformation takes place after the first of the three methyl groups has been removed from lanosterol. A confirmatory result is that 4,4-dimethyl-$\Delta^{8,24}$-cholestadiene-3-one is a precursor of cholesterol while lanostadienone is not (12). If 4-carboxy sterols are intermediates, as we suspect, then the presence of a keto group at the 3 position β to the carboxy group can be looked upon as a means of facilitating decarboxylation. It can be argued, on the other hand, that the methyl group at C-14 is too distant from the 3-oxygen function, and hence that in this case the necessary activation is provided by the 8,9-double bond.

Zymosterol has the same relatively rare $\Delta^{8,24}$-double bond system as lanosterol, and it has therefore been logically regarded as the first fully demethylated cholesterol precursor. Zymosterol can be readily isolated from yeast, and though the evidence that it is a normal constituent of animal tissues is not yet very strong, this sterol shows the metabolic activity expected from a normal cholesterol precursor (10, 20). The final structural alteration beyond zymosterol (assuming this to be an obligatory intermediate) cannot be formulated with any assurance except that the occurrence and metabolic activity of desmosterol, $\Delta^{5,24}$-cholestadienol (21), strongly point to the reduction of the side chain double bond as the last step in cholesterol biogenesis. It is worth noting that the over-all change from zymosterol to cholesterol, though it furnishes a more reduced product, nevertheless is dependent on molecular oxygen (10). As a mechanism for relocating the nuclear double bond from the 8,9 to the 5,6 position, a simple isomerization is therefore ruled out. The requirement for oxygen conceivably reflects the introduction of an additional hydroxy group in ring B, most likely in allylic

position in which case the 5 6 double bond would be newly introduced and established by elimination of water

REFERENCES

1 Amdur B H Rilling H and Bloch K *J Am Chem Soc* **79** 2646 (1957)
2 Bloch K *Vitamins and Hormones* **15** 119 (1957)
3 Bucher N L R and McGarrahan K *J Biol Chem* **222** 1 (1956)
4 Chaykin S and Bloch K (1957)
5 Djerassi C Mills J S and Villotti R *J Am Chem Soc* **80** 1006 (1958)
6 Eschenmoser A Ruzicka L Jeger O and Arigoni J *Helv Chim Acta* **38** 1890 (1955)
7 Ferguson J J Durr I F and Rudney H *Federation Proc* **17** 219 (1958)
8 Gautschi F and Bloch K *J Am Chem Soc* **79** 684 (1957)
9 Gautschi F and Bloch K unpublished results
10 Johnston J D and Bloch K *J Am Chem Soc* **79** 1145 (1957)
11 Langdon R G *J Biol Chem* **226** 615 (1957)
12 Lindberg M Gautschi F and Bloch K (1957)
13 Mudgal R K Tchen T T and Bloch K *J Am Chem Soc* **80** 2580 (1958)
14 Mazur Y Weizmann A and Sondheimer F *J Am Chem Soc* **80** 1009 (1958)
15 Olson J A Lindberg M and Bloch K *J Biol Chem* **226** 941 (1957)
16 Phillips A H Tchen T T and Bloch K *Federation Proc* **17** 289 (1958)
17 Rilling H Tchen T T and Bloch K *Proc Natl Acad Sci U S* **44** 167 (1958)
18 Rudney H *J Biol Chem* **227** 663 (1957)
19 Ruzicka L Eschenmoser A and Heusser H *Experientia* **9** 357 (1953)
20 Schwenk E Alexander G J Fish C A and Stoudt T H *Federation Proc* **14** 752 (1955)
21 Stokes W M Fish W A and Hickey F C *J Biol Chem* **220** 415 (1956)
22 Tavormina P A Gibbs M H and Huff J W *J Am Chem Soc* **77** 4498 (1956)
23 Tchen T T *J Am Chem Soc* **79** 6345 (1957)
24 Tchen T T and Bloch K *J Am Chem Soc* **78** 1516 (1956)
25 Tchen T T and Bloch K *J Biol Chem* **226** 931 (1957)
26 Wells W W and Neiderhiser D H *J Am Chem Soc* **79** 6569 (1957)
27 Wolf D E Hoffman C H Aldrich P E Skeggs H R Wright L D and Folkers K *J Am Chem Soc* **77** 4499 (1956)

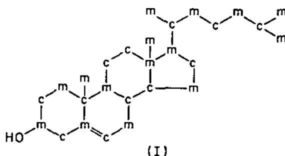
Some Aspects of the Biosynthesis of Cholesterol from Mevalonic Acid

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This report is the result of the joint effort of our team consisting of J W Cornforth Rita H Cornforth Irene Youhotsky Gore L Gosselin G Popjak and A de Waard Detailed accounts of most of the experiments are contained in two papers to be published shortly in the *Biochemical Journal* (3 9) Summaries of the results have already appeared (2 6 8)

When two years ago we completed our studies on the distribution of acetate carbons in the ring structure of cholesterol biosynthesized from C^{14} acetate (4) we could assign every carbon atom in the sterol to either the methyl or the carboxyl carbon of acetate [formula (I)] The pattern shown in formula (I) was in complete accord with the Woodward Bloch hypothesis of cyclization of squalene to sterol

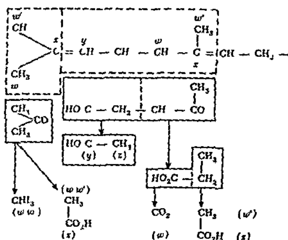


(I)

In spite of a great deal of experimental work trying to implicate branched chain C 6 and C 5 acids such as 3 hydroxy-3 methylglutarate (HMG) 3 methylglutaconate *iso* valerate 3 hydroxy*iso* valerate 3 methylcrotonate (dimethylacrylate) as the source of isoprenoid units used in the biosynthesis of squalene and sterol no definite proof to this effect could be obtained The situation was however changed very dramatically with the discovery of mevalonic acid (10) and identification of its structure as 3 hydroxy 3 methylpentano-5 lactone (13 14) As you are all aware the similarity of the structure of mevalonic acid (MVA) to that of HMG prompted Tavormina *et al* (11) to test this

new substance as a precursor of cholesterol Their discovery that DL 2 C^{14} MVA was utilized for the biosynthesis of cholesterol in liver homogenates with an efficiency of about 40% made it very probable that at last the direct source of isoprenoid units had been found We thought that a proof of this could be obtained in the surest way by ascertaining first whether squalene was also synthesized from MVA and if so by determining the arrangement of the MVA carbons in squalene

The news of the discovery of Tavormina *et al* (11) reached us in England early in November of 1956 but since the methods for the synthesis of MVA were not published at that time we had to develop these for ourselves Dr and Mrs Cornforth worked out the synthesis



SCHEME 1 Degradation of squalene into acetone and levulinic acid and into further fragments Only the first two isoprenoid units from one half of the squalene molecule are shown

not only of MVA but of all the anhydro compounds derivable from it they were all labeled with C^{14} in position 2 and MVA in position 1 also The anhydro compounds were made in order to test some of our ideas on the possible transformations of MVA during the biosynthetic reactions I will discuss briefly the experiments with these substances at the end of my communication We have been able to confirm the results of Tavormina and associates (11) without any difficulty and to show that liver homogenates under anaerobic conditions synthesized only squalene from 2-C^{14} MVA Moreover, the efficiency of squalene synthesis from MVA anaerobically was as great as the synthesis of cholesterol aerobically This result was very satisfying because it supported fully the view that squalene was an intermediate in sterol bio

synthesis and it also enabled us to prepare a large batch of C^{14} squalene for chemical degradation

We degraded the sample of squalene biosynthesized from 2 C^{14} MVA by ozonolysis as described previously (5) Acetone levulinic acid and succinic acid are the principal products of ozonolysis of squalene of these only acetone and levulinic acid can be relied upon as arising from carbon atoms predicted by theory The levulinic acid contains all five types of carbon atoms of the isoprenoid units of squalene and consequently its carbon by carbon degradation gives an answer as to the arrangement of isotopic carbon in squalene (Scheme 1)

TABLE I

DISTRIBUTION OF C^{14} IN SQUALENE BIOSYNTHESIZED FROM 2-C^{14} MEVALONIC ACID

Compounds and fragments analyzed	Specific activity of total carbon counts/min at infinite thickness (A)	Molar specific activity[a] ($A \times n$)	
Squalene	788 ± 39	3940 × 6	
Acetone ($u + x + w'$)[b]			
Methyl carbons (w w')	1967 ± 98	1967	4047
Acetic acid ($w + x$ $w' + x$)	1040 ± 52	2080	
Levulinic acid ($w' + x + w + z + y$)			
4 Aminopentanoic acid	777 ± 39	3890	
Acetic acid(1) ($z + y$)	0	0	
Propionic acid ($w + x + w$)	1323 ± 66	3975	
COOH of propionic acid (w)	3880 ± 194	3880	
Acetic acid( ) ($w + x$)	0	0	

[a] This was obtained by multiplying the values in Column A by the number (n) of carbon atoms contained in the compound analyzed The molar specific activity of squalene is given as a multiple of 6 since squalene contains 6 isoprenoid units

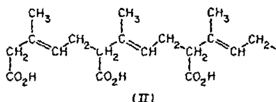
[b] The letters in parentheses indicate the carbon atoms of isoprenoid units (cf Scheme 1)

The results of such degradation are shown in Table I It is seen that the molar specific activity of the acetone was equal to one sixth of the molar specific activity of the squalene Since the specific activity of the acetic acid obtained from the acetone after the iodoform reaction and which contained equally carbons ($w + x$) and ($w' + x$) was one half of that of the methyl carbons of acetone (w w') carbon atom x contained evidently no C^{14} The radioactivity of acetone is therefore either distributed between w and w' or is contained in only one of these carbons

The levulinic acid which was assayed both as the 2 4 dinitrophenyl

hydrazone and the 4 aminopentanoic acid had a molar specific activity also one sixth of that of squalene All the C^{14} in the levulinic acid was confined to one carbon atom *w* which is C-3 of this acid and appeared as the carboxyl carbon of propionic acid during degradation

These results can mean only that squalene biosynthesized from 2 C^{14} MVA contains six labeled positions one in each of the two terminal methyl groups and four within the carbon chain of the hydro carbon corresponding to carbon atom *w* of the isoprenoid units Al though by the degradation of acetone we cannot distinguish between carbons *w* and *w* the analysis of levulinic acid showed that only carbon atom *w* contained C^{14} hence we infer that even in the terminal methyl groups only one atom is labeled The mevalonic acid therefore must have given rise to an isoprenoid unit which is asymmetrically labeled and which without further degradation underwent polymerization to squalene That the two terminal methyl groups of the isoprenoid units undergoing condensation to squalene have remained asymmetrically labeled is open to two interpretations First it is possible that MVA itself or another 6 carbon compound derived from it is the condensing unit C 5 of one unit being linked to C 2 of another such mechanism leads to a hypothetical product containing not only branched methyl but also branched carboxyl groups (formula II) the latter being re



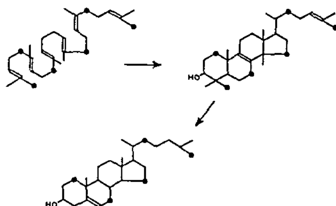
(II)

moved afterward The second possibility is that the carboxyl carbon of MVA is lost before the condensation to leave a C 5 branched chain compound Such a compound might even have two apparently in distinguishable methyl groups and yet be treated by enzymes in an *asymmetric manner*

The cyclization of squalene to sterol (lanosterol) proceeds un doubtedly according to the general scheme put forward by Woodward and Bloch (15) Thus the labeled positions in lanosterol and in cho lesterol biosynthesized from 2 C^{14} MVA may be predicted to be as shown on Scheme 2 and have already been confirmed by Isler and his colleagues (7) by partial degradation of C^{14} cholesterol biosynthesized from 2 C^{14} MVA

Components of Liver Homogenates and Coenzymes Required for Squalene and Sterol Synthesis from Mevalonic Acid

We have devoted some attention to the liver enzyme system synthesizing squalene and sterol from MVA in the belief that an intimate knowledge of the enzymic reactions will provide the answers to the still unsolved problems As a first step we examined the components of homogenates needed for sterol synthesis We learned very soon that the mitochondria can be removed from the homogenates without impairing the synthetic activity of the preparations and therefore we concentrated our attention on fractionation of preparations from which

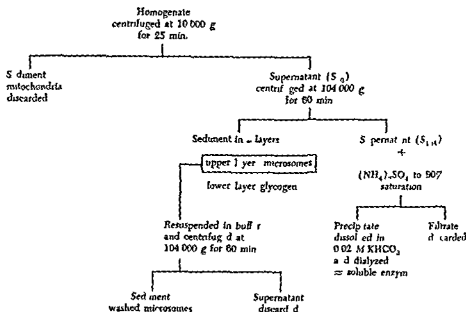


SCHEME 2 Labeling of squalene and sterols biosynthesized from 2 C[14] mevalonic acid Labeled positions are marked with black circles

the mitochondria have been removed by centrifugation at 6000 g or 10 000 g for 25 minutes (S_6 or S_{10} preparations see Scheme 3) When either an S_6 or S_{10} preparation is centrifuged at a mean force of 104 000 g for 1 hour the following layers are obtained (*1*) a thin pad of fat at the top (*2*) the supernatant of soluble proteins (S_{104}) and (*3*) a sediment in two layers the lighter one is the microsomal layer and the heavier one is almost pure glycogen

Supernatants of homogenates centrifuged at 6000 10 000 or 30 000 g contain all the components required for the synthesis of squalene or sterol The enzymes of this system are contained in the soluble protein (S_{104}) and the microsomal fraction the glycogen rich sediment is not required

Although the S_{104} fraction by itself could not form sterol it could synthesize some squalene from MVA but very poorly in comparison with the full system containing microsomes



SCHEME 3 Fractionation of liver enzyme preparations

It was our general experience that the fresh S_{104} preparations combined with microsomes possessed maximal activity for sterol synthesis and that addition of known coenzymes such as diphosphopyridine nucleotide (DPN) its reduced form (DPNH) and adenosinetriphosphate (ATP) caused no stimulation on the contrary they often proved slightly inhibitory There were a few exceptions to this rule as is shown by the experiment of Table II in which the enzyme preparation showed

TABLE II

SYNTHESIS OF STEROL FROM MVA BY FRACTIONS OF LIVER HOMOGENATE EXPERIMENT SHOWING NEED FOR PYRIDINE NUCLEOTIDES[a]

Additions	Specific activity of sterol digitonides counts/min at infinite thickness
None	188
DPNH[b] (0.5 µmole)	920
DPNH[b] + TPNH[c] (0 5 µmole)	3780

[a] Each flask contained 2 0 ml of S_{104} (34 1 mg protein/ml) suspension of washed microsomes 0 2 ml and 2 C^{14} MVA 0 5 µmole (0 1 µc C^{14}) Additions were as shown Final volume 4 0 ml made up with standard buffer Incubation at 37 C for 1 hour

[b] Kept reduced with alcohol dehydrogenase (0 6 mg) + ethanol (5 µmoles)

[c] Reduced with glucose 6 phosphate (4 µmoles) S_{104} contains glucose 6-phosphate dehydrogenase in nonrate limiting amounts

very poor synthetic activity without added DPNH and TPNH (reduced form of triphosphopyridine nucleotide) The need for pyridine nucleotides could be shown in a more convincing way by using the dialyzed soluble enzyme (S) reconstituted after ammonium sulfate precipitation (cf Scheme 3) in combination with microsomes Such an enzyme system did not synthesize sterol without addition of coenzymes some of which had to be provided in the form of a boiled protein free extract of fresh S_{104} Occasionally we made such boiled extracts that seemed to contain all the necessary coenzymes but generally pyridine nucleotides had to be added as well as the boiled extract to reactivate the

TABLE III

NEED FOR PYRIDINE NUCLEOTIDES IN STEROL SYNTHESIS FROM MVA[a]

Additions	MVA converted into sterol (μmole)
None	0 000
B E[b] (2 0 ml)	0 005
B E + DPN (0 5 μmole)	0 055
B E + DPNH (0 5 μmole)	0 119
B E + ATP (5 μmoles)	0 005
B E + TPN (0.5 μmole)	0 077

[a] Each flask contained soluble dialyzed enzyme (S) 1 ml (21.5 mg protein/ml) suspension of washed microsomes (0 2 ml) and 2 C^{14} MVA 0.5 μmole Additions were as shown Final volume 3 5 ml made up with standard buffer Incubation at 37 C for 1 hour

[b] B E = boiled protein free extract of S_{104}

TABLE IV

NEED FOR PYRIDINE NUCLEOTIDES IN STEROL SYNTHESIS FROM MVA[a]

	Additions	MVA converted into sterol (μmole)
1	Control	0 219
2	B E[b]	0 000
3	B E + DPNH	0 097
4	B E + TPNH	0 025
5	B E + DPNH + TPNH	0 153
6	DPNH + TPNH + ATP	0 005

[a] The control flask (No 1) contained fresh S_{104} (2 5 ml 24 mg protein/ml) + 0 2 ml microsomes + DPNH (0 5 μmole) The experimental flasks (Nos 2–6) contained dialyzed soluble enzyme (S) 1 0 ml (40 5 mg protein/ml) + 0 2 ml washed microsomes Additions to experimental flasks were boiled extract[b] (2 ml) DPNH (0 5 μmole) reduced with alcohol dehydrogenase and ethanol TPNH (0 5 μmole) reduced with glucose-6-phosphate (2 μmoles) and the specific dehydrogenase ATP 2 5 μmoles All flasks contained 0 5 μmole of 2 C^{14} MVA The final volume was 3 5 ml made up with standard buffer Incubation for 1 hour at 37 C

[b] B E = boiled protein free extract of S_{104}

enzyme system We found that DPNH rather than DPN was required but triphosphopyridine nucleotide (TPN) could replace DPN However for maximal activity both DPNH and TPN were needed (Tables III and IV) In experiments in which we had to use the boiled extract of S_{104} as a source of some of the cofactors we could not at first decide whether ATP was an essential component or not if ATP was needed it must have been present in our preparations in sufficient amounts to promote the synthesis

That the boiled extracts contained some factor other than ATP was shown by the fact that it could not be replaced by pyridine nucleotides plus ATP After trying several substances [adenosine monophosphate (AMP) adenosine diphosphate (ADP) guanosine triphosphate (GTP) cytidine triphosphate (CTP) uridylic acid (UMP) lipoic acid, diphosphothiamine CoA SH $MnCl_2$] without success to replace the boiled extract of S_{104} we found that reducing agents such as cysteine glutathione cysteamine or ascorbate could substitute for the boiled extract (Table V) These boiled extracts varied in potency

TABLE V

COENZYME REQUIREMENTS IN STEROL SYNTHESIS FROM MVA[a]

Additions	Specific activity of sterol digitonides counts/min at infinite thickness
B E[b]	85
B E[b] + DPNH + TPNH	1200
DPNH + TPNH + ATP	173
DPNH + TPNH + ATP + CoA	123
DPNH + TPNH + ATP + CSH	2620
DPNH + TPNH + ATP + CSH + CoA	3060[c]
DPNH + TPNH + CSH + CoA	8

[a] Each flask contained soluble dialyzed enzyme (S) 1 0 ml (40 1 mg protein/ml) washed microsomes 0 2 ml 2 C^{14} MVA 0 5 μmole Additions as shown were boiled extract (B E) 2 0 ml DPNH and TPNH 0 5 μmole (both reduced with specific dehydrogenase and substrate) CoA 0 5 μmole ATP 5 μmoles cysteine 30 μmoles Final volume 4 ml made up with standard buffer Incubations for 1 hour at 37 C

[b] B E = boiled protein free extract of S_{104}

[c] Equivalent to a conversion of 0 09 μmole of MVA into sterol

a great deal—apart from being deficient in pyridine nucleotides—and this variability could be correlated with their content of free SH groups extracts with high free SH content (3–5 μmoles/ml) were always the most potent in activating sterol synthesis Out of the four reducing agents, glutathione and ascorbate seemed to be the most effective The

addition of CoA to these reducing agents normally produced no more than 15–25% stimulation In the absence of a boiled extract the absolute dependence of sterol synthesis on ATP came to light also (cf Table V)

In the enzyme system activated with glutathione (GSH) in the presence of air both squalene and sterol are formed from 2 C^{14} MVA most of the C^{14} appearing in sterol under N however only squalene is synthesized and the addition of GSH is not necessary Aerobically neither squalene nor sterol is formed if the reducing agent is omitted from the incubation (Table VI)

TABLE VI
EFFECTS OF GLUTATHIONE AND GAS PHASE ON SYNTHESIS OF SQUALENE AND STEROL FROM 2 C^{14} MVA

Addition	Gas phase	Total C^{14} counts found in	
		Squalene	Sterol digitonides
None	Air	0	0
GSH	Air	5 130	15 771
None	N_2	44 000	0
GSH	N	48 690[b]	1,288

[a] Standard incubation mixtures with 0 5 μmole of 2 C^{14} MVA (0 1 μc C^{14}) Each flask contained 10 mg of squalene from start Addition of glutathione (GSH) 30 μmoles as shown In the anaerobic experiments the flasks were being flushed through continuously with N

[b] Corresponds to a utilization of 0 19 μmole of MVA

In order to decide whether the enzyme or coenzyme protected by the reducing agents is involved in the reactions preceding the formation of squalene or in the cyclization of the latter to sterol a set of experiments was carried out in which the enzyme system was incubated with all the cofactors but without a reducing agent for 1 hour under N (Table VII) During this time synthesis of squalene alone was expected to occur Then the N was replaced by air and the incubation continued for another hour When pyridine nucleotides were not added at the beginning of the aerobic period (2nd hour) only a small amount of radioactive sterol appeared even if GSH had been added at that time When the addition of DPNH and TPN was repeated on admission of air an appreciable amount of squalene formed during the anaerobic period was converted into sterol during the 2nd hour even in the absence of GSH or ascorbic acid The conclusion to be drawn from the last two experiments is that GSH or ascorbate protect from oxidation an enzyme or coenzyme involved in the reactions leading to the synthesis of squalene from MVA and not in the cyclization of squalene to sterol

TABLE VII

SYNTHESIS OF SQUALENE AND OF STEROL FROM MVA IN THE ABSENCE OF REDUCING AGENT[a]

Gas phase and additions during				Total C^{14} (counts/min) found in	
1st hour		2nd hour		Squalene	Sterol digitonides
Experiment A					
N	None	Air	None	20 920[b]	231
N	None		—[c]	23 550	0
N_2	None	N	None	22 310	0
N_2	None	Air	GSH	15 920	847
Air	GSH	Air	None	1610	14 126
Experiment B					
N	None	Air	None	9870	337
N	None	Air	DPNH + TPN	9240	3870
N	None	Air	DPNH + TPN + ascorbate	6820	4956

[a] Each flask contained the standard incubation mixture with 0.5 μmole of 2 C^{14} MVA (0 1 μc C^{14}) Additions of GSH and of ascorbate (30 μmoles each) DPNH (1 μmole) and of TPN (0 5 μmole) were made as shown During the anaerobic period of incubations the flasks were being flushed continuously with N_2 Experiments A and B were done separately with different enzyme preparations neither of which was particularly active

[b] Corresponds to conversion of 0 08 μmole of MVA

[c] Incubation for 1 hour only

EFFECT OF SH INHIBITORS ON SQUALENE AND ON STEROL SYNTHESIS FROM MVA

As it seemed probable that the oxygen sensitive substance in the enzyme system was an SH compound we investigated the effects of HgCl and of p chloromercuribenzoate (PCMB) on the anaerobic synthesis of squalene Both mercurials in a concentration of 10^{-3} M almost completely abolished the formation of squalene As might be expected GSH but not ascorbate partly counteracted the inhibition caused by PCMB

We have been able to show that the components of the enzyme system sensitive to PCMB were present mainly in the microsomes For this purpose the standard amount of microsomes (0 2 ml) was added to 2 ml of buffer containing 4 μmoles of PCMB the mixture was left standing in ice for 3 minutes thereafter 30 μmoles of GSH were added, followed after 1 minute by DPNH TPN ATP and 1 ml of soluble enzyme and MVA The mixture was then incubated aerobically for 1 hour The formation of sterol in this system was compared to that in another system in which the soluble enzyme (1 ml) was treated with

PCMB (4 μmoles) for 3 minutes at 0°C the addition of GSH DPNH TPN ATP and untreated microsomes and MVA (in that order) completed the system As is shown in Table VIII there was nearly complete failure of sterol synthesis in the system containing the pretreated microsomes in the incubation containing the pretreated soluble enzyme only 50% inhibition was observed

TABLE VIII
THE EFFECT OF SH INHIBITORS ON STEROL SYNTHESIS AND LOCALIZATION OF INHIBITED SUBSTANCE IN THE MICROSOMES[a]

	Experimental conditions	Specific activity of sterol digitonides counts/min at infinite thickness
1*a*	Control	3430
1*b*	Control + CoA	4611
2*a*	Soluble enzyme pretreated with PCMB	1859
2*b*	Same as 2*a* + CoA	2286
3*a*	Microsomes pretreated with PCMB	217
3*b*	Same as 3*a* + CoA	49

The control flasks (1*a* and 1*b*) contained standard incubation mixture with 0 5 μmole of 2 C^{14} MVA (0 1 μc C^{14}) and 30 μmoles of GSH In flasks 2*a* and 2*b* the soluble enzyme and in flasks 3*a* and 3*b* the microsomes were pretreated with 4 μmoles of PCMB otherwise all the components were the same as in the control CoA (0 5 μmole) was added as shown Final volume 4 ml in all flasks For details of experiment see text Incubations at 37 C for 1 hour gas phase air

It is worth considering for a moment the implications of the latter results clearly showing the participation of an SH compound in the synthesis of squalene from MVA The requirement for a reducing agent for activation is reminiscent of all reactions dependent on coenzyme A From all the accumulated evidence it is difficult to see at present what role we could assign to CoA in these reactions as it does not seem probable that the decarboxylation of MVA should require carboxyl activation likewise in the condensation of C 5 of one MVA unit to the C 2 of another a Claisen type of condensation—involving the previous oxidation of the C 5 group to carboxyl—has been excluded by the results of Amdur *et al* (1) obtained with 5 tritio 2 C^{14} MVA Perhaps the requirement for the reducing agent under aerobic conditions may be attributed to the participation of a highly sensitive SH enzyme but we would like to keep an open mind on this question at present Certainly our enzyme system—even after dialysis—contains some coenzyme A as judged by activation of acetate

It is worth mentioning here that we obtained no evidence as to the

possible breakdown of mevalonic acid to acetate as judged by the complete absence of radioactivity from acethydroxamate which we isolated after incubation of S_{10} preparations with 2 C^{14} MVA

Optimum Condition for Sterol Synthesis from MVA

We examined the optimum pH and optimum concentration of the various coenzymes for sterol synthesis by our liver enzyme preparations on the basis of these experiments a standard incubation mixture was worked out This consisted of dialyzed soluble enzymes 1 ml (30-50 mg protein) washed microsomes 0 2 ml DPNH 1 μmole TPN, 1 μmole ATP 10 μmoles K phosphate buffer pH 7 4 400 μmoles nicotinamide 120 μmoles $MgCl$ 16 μmoles MVA 0 5-2 μmoles GSH or ascorbate 30 μmoles in a final volume of 4 ml

Preservation of Enzymes and Microsomes

As far as we can tell the soluble enzymes may be preserved for at least several months at —15 C in the form of a sludge precipitated with ammonium sulfate These enzymes can be reconstituted by dissolving in 0 02 *M* $KHCO_3$ (3 g sludge in 12 ml $KHCO_3$) followed by dialysis Also they can be partially purified by fractionation with ammonium sulfate all the enzymic activity is found in the fraction precipitating between 30-60% saturation the removal of soluble ribonucleic acid (RNA) with protamine from this fraction does not impair in any way the enzymic activity

The preservation of the microsomes presented some problems and until recently we had to prepare microsomes freshly on the day of each experiment We had a very curious experience with microsomes stored at either —15 or at —79 C When these were tested in combination with freshly prepared undialyzed S_{104} microsomes stored for 1 month seemed to have lost only about one half of their activity but when we tried them with our reconstituted system of the standard incubation mixture containing the dialyzed soluble enzymes there was virtually no synthetic activity detectable Fresh microsomes on the other hand were always active with the reconstituted system After many trials and failures to keep the microsomes alive we found that even the most ancient of our microsome preparations (the oldest so far tested was 4 months old) can be rejuvenated by the addition of Mn^{++} in a final concentration of 10^{-3} *M* When the amount of ATP in the incubations is increased from 10 to 30 μmoles in the presence of 10^{-3} *M* Mn^{++} one can no longer tell the difference between systems containing fresh microsomes or those containing microsomes kept for several months at —15°C We hope that this observation will be of some help to us and to others

also working in this field Certainly Mn^{++} must be considered as an other cofactor of the liver enzyme system

FORMATION OF INTERMEDIATES FROM MVA

The formation of new substances derived from MVA in enzyme incubations was first observed nearly a year ago on paper chromatograms of the deproteinized incubations of S_{10} preparation with 2 C^{14} MVA or with 1 C^{14} MVA At least two substances more polar than MVA still containing C 1 of MVA have so far been observed Of these one has been isolated after chromatography on Dowex 2 (formate) ion exchange resin and was found to be utilized at least as efficiently as MVA for sterol synthesis

When a standard incubation mixture containing no enzymes but all the cofactors and either 1 C^{14} MVA or 2 C^{14} MVA is treated with perchloric acid (0 5 *M*) and then neutralized with KOH to remove perchlorate the perchlorate free filtrate gives a chromatogram on Whatman No 1 paper (isobutyric/ammonia/H O solvent system) as shown in Fig 1*a* There are two large spots and a small one absorbing ultraviolet light and two partially separated radioactive spots (R_f 0 75–0 80 and 0 65–0 69) which do not coincide with the ultraviolet absorbing spots These two radioactive areas represent an equilibrium mixture of mevalonic lactone and acid after isolation they are readily convertible into one another After incubation with the enzyme system for 5 minutes additional ultraviolet absorbing spots appear (Fig 1*b* and *c*) at least one of which also contains radioactivity The large ultraviolet absorbing and radioactive spot has always a delicate pink color

When the deproteinized incubation mixture is put on Dowex 2 (formate) resin and gradient elution is carried out with increasing concentrations of formic acid and ammonium formate three radioactive substances emerge (Fig 2) Peaks I and II are mevalonic acid and lactone identical with the fast moving substances found on paper chromatograms after 20-minutes incubation these are biologically completely inactive and presumably are either the D or L forms of MVA Peak III which emerges only after ammonium formate has been introduced into the column is identical with the large polar ultraviolet absorbing radioactive spot found on the paper chromatograms It is used by the liver enzyme system for sterol synthesis at least as efficiently as MVA but it still requires the addition of ATP to the enzyme system When rechromatographed on paper with isobutyric/ammonia/water butanol/acetic acid/water or butanol/formic acid/water solvent systems the radioactivity and ultraviolet absorbing materials still remain together (Fig 1*d*) However with saturated ammonium sulfate/Na

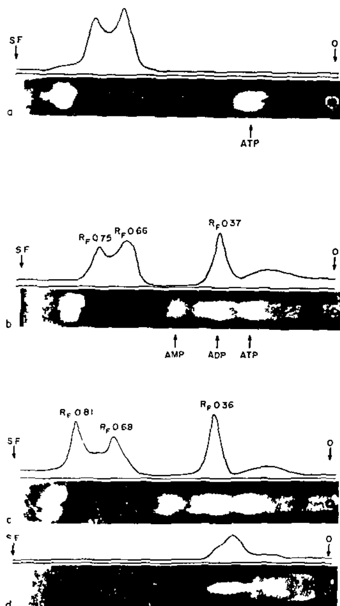


FIG 1 Paper chromatograms showing formation of new derivative of mevalonic acid The black strips with white spots are photographic prints made with ultra violet light of the chromatograms the curves above these are records of radioactive scanning of the chromatograms For description see text

acetate/isopropanol or with methanol/ammonia/water solvent systems the radioactivity is separated from the ultraviolet absorbing material the latter appears to be identical with ADP The radioactive substance freed from ADP is not mevalonic acid or lactone but a more polar derivative of it it contains all six carbon atoms of MVA We do not know yet if this substance is identical with the phosphorylated derivative of MVA the formation of which has been reported by Tchen (12)

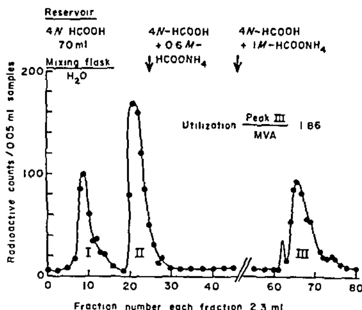


FIG 2 Chromatography on Dowex 2 (formate) of substances derived from mevalonic lactone Peak III is a new derivative formed by liver enzymes in the presence of ATP

The new derivative of MVA is formed by the soluble liver enzyme fraction alone in the presence of ATP (cf Fig 1*b*) The formation of ADP but of very little AMP suggests that inorganic orthophosphate is split off from ATP during the incubations with MVA

As I have indicated at the beginning we have prepared all the anhydro compounds (labeled with C^{14} on C 2) derivable from MVA (*1*) 3 methylpent 2 eno 5 lactone and the *cis* 5 hydroxy-3 methylpent 2 enoic acid (*2*) *cis*-3 methylpenta 2 4 dienoic acid (*3*) 3 hydroxy-3 methylpent 4 enoic acid (*4*) a mixture of *cis* and *trans*-3 methylpenta 2 4 dienoic acid (*5*) a mixture of *cis* and *trans* 5 hydroxy-3 methylpent 3 enoic acid and (*6*) most recently we have also obtained a specimen containing the mixture of four acids the *cis* and *trans* isomers of 5

hydroxy-3 methylpent 2 enoic and of 5 hydroxy-3 methylpent-3 enoic acids from this mixture the two *trans* acids (*trans* 5 hydroxy-3 methyl pent 2 enoic and pent 3 enoic acids) have been separated We went to all this trouble because like Dr Bloch we envisaged that by dehydration and decarboxylation mevalonic acid could yield isoprene whose polymerization can be visualized readily as proceeding by electron shifts induced by the attack of a cation requiring the net reduction of only two double bonds to form squalene

However all the unsaturated compounds labeled with C^{14} in position 2 and related to MVA gave negligible labeling of sterol when incubated with our S_{10} preparation Neither could we obtain evidence as to the formation of isoprene from 2 C^{14} MVA although isoprene suppressed the incorporation of MVA into cholesterol to one half of the control value

The negative results with the unsaturated analogs of mevalonic acid may mean that none of these acids is an intermediate formed from MVA or that an enzyme required for activation is lacking Taking the first assumption to be correct one is led almost inevitably to the conclusion that the condensation of mevalonic units the elimination of OH groups and of C 1 of MVA proceeds by the type of concerted reaction just discussed by Dr Bloch

REFERENCES

1 Amdur B H Rilling H and Bloch K *J Am Chem Soc* 79 2647 (1957)
2 Cornforth J W Cornforth R H Popjak G and Gore I Y *Biochem J* 66 10P (1957)
3 Cornforth J W Cornforth R H Popjak G and Gore I Y *Biochem J* 69 146 (1958)
4 Cornforth J W Gore I Y and Popjak G *Biochem J* 65 94 (1957)
5 Cornforth J W and Popjak G *Biochem J* 58 403 (1954)
6 Gould R G and Popják G *Biochem J* 66 51P (1957)
7 Isler O Ruegg R Wursch J Gey K F and Pletcher A *Helv Chim Acta* 40 2369 (1957)
8 Popjak G Gosselin L and Gore I Y *Biochem J* 68 15P (1958)
9 Popjak G Gosselin L Gore I Y and Gould R G *Biochem J* 69 238 (1958)
10 Skeggs M R Wright L D Cresson E L MacRae G D E Hoffman C H Wolf D E and Folkers K *J Bacteriol* 72 579 (1956)
11 Tavormina P A Gibbs M H and Huff J W *J Am Chem Soc* 78 4498 (1956)
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14 Wolf D E Hoffman C H Aldrich P E Skeggs H R Wright L D and Folkers K *J Am Chem Soc* 79 1486 (1957)
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DISCUSSION OF PAPERS BY DRS. BLOCH AND POPJÁK

WHITE: Dr Bloch was of the opinion, I think, that there is a hydrolysis of the postulated mevalonic diphosphate to isoprene prior to condensation. Dr Popják stated that he was unable to find isoprene in their incubation mixture. I wonder why Dr Bloch has rejected the idea of a possible diphosphate diester as the actual unit undergoing condensation, with elimination of pyrophosphate.

BLOCH: I believe I talked in terms of phosphate elimination rather than hydrolysis of the phosphate ester. If you eliminate a phosphate anion, you introduce a double bond directly. As far as isoprene is concerned, we have in fact prepared labelled isoprene and found it to be totally inactive as a precursor. We do not think that this result is necessarily in conflict with our postulated scheme, provided we do not insist on free isoprene as an intermediate. What I have presented is—or so it appears to us—the simplest mechanism to fit the isotopic data. For example, the D_2O data force us to conclude that one of the two phosphates is split off simultaneously with the decarboxylation and before condensation occurs. It is also conceivable that the monoenol phosphate is the actual condensing unit. This possibility remains open.

GOULD: In connection with Dr Bloch's suggestion that, in the final stages of cholesterol biosynthesis, the double bond may be removed and then a new one formed in the 5-6 position, I would like to ask if he has any suggestions about why it is that dihydrocholesterol cannot be converted into cholesterol. Are there specific structural requirements for the formation of the 5-6 double bond?

BLOCH: I think I was not very clear on this point. I raised the possibility that it may not be the 8-9 double bond which becomes the 5-6 double bond on the grounds that, for example, 7-dehydrocholesterol is converted to cholesterol. This suggests the following sequence: a shift of the 8-9 double bond to the 7-8 position, an oxidation at C-6 to yield a 6-hydroxy derivative, elimination of water to 7-dehydrocholesterol, or more likely the $\Delta^{5,7,24}$-unsaturated sterol which would be a trienol, and finally the reduction of the 7-8 double bond to desmosterol.

POPJÁK: The problem in what sequence the movement of the double bond occurs in cholesterol precursors is an extremely difficult one. The isolation and identification of 4α-methylcholest-7-enol raised the question why is the double bond in zymosterol still in the 8-9 position? You were referring, no doubt, to the substance Djerassi isolated from the cactus?

BLOCH: Yes, and also to the 4α-methyl sterols isolated by Wells from rat feces and by Sondheimer from citrus.

POPJÁK: I have seen Djerassi's note and I think it is quite clear that his compound (lophenol) is not identical with the substance isolated by Wells from the feces of rats. The physical properties of the two are so different that they cannot be identical. The compound isolated from the cactus is definitely 4α-methylcholest-7-enol. Now if that is really one of the demethylation products of the 14-norlanosterol, then how does zymosterol still retain the double bond in the 8-9 position? It is very difficult really to decide the sequence of these events. Now about your proposed mechanism of the condensation of farnesene to squalene: if the proton attack and hydride elimination is the mechanism of the condensation of the two molecules and two atoms of hydrogen are introduced, then in the squalene so formed you should get a substantial amount of deuterium or tritium which should appear in levulinic acid obtained by ozonolysis of squalene. The two methyl carbons in question will be the methyls of levulinic acid. Did you observe this?

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BLOCH As you may recall we have small amounts of heavy hydrogen in the succinic acid from the central carbon atoms of squalene and we have also very small amounts of deuterium atoms in the levulinic acid There is an ambiguous situation here because the proton and the hydride ion can attack at different positions depending on the structure which you assign to farnesene As I pointed out the position of the diene system in farnesene is not established I would like to ask Dr Schwenk's opinion about zymosterol as an obligatory intermediate

SCHWENK We have not made more experiments with zymosterol than those which we have published so I cannot add anything As to experiments with mevalonic acid I would like to point out that in all of the published experiments with this substance the sterols were isolated with digitonin and counted as digitonides It was not investigated whether the cholesterol was pure We have given mevalonic acid 2 C^{14} obtained through the courtesy of Merck & Co intraperitoneally to rats and isolated the sterols as usual from the liver the gastrointestinal tract and from

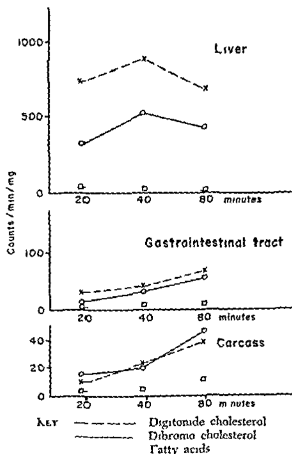


FIG A Male Sprague Dawley rats weighing 180 g were injected intraperitoneally with 0 2 μc of mevalonic acid 2 C^{14} as the sodium salt They were killed at the indicated time intervals and the tissues hydrolyzed with 30% KOH Isolation and purification of sterols see *Arch Biochem Biophys* 42 91 1953

the carcass 20, 40 and 80 minutes after the injection. Figure A shows the results obtained. Incorporation into the sterols is rapid and reaches a comparatively high degree after 40 minutes. *A control experiment with the same amount of C^{14} in the form* of acetate gave only traces of radioactivity in the cholesterol. The most important observation is however the large amount of high counting companions (HCC) in the sterols from the digitonides. Bromination of these sterols proves that they contain 40–50% of the counts in substances other than cholesterol and the course of the two curves shows that only parts of these HCC are precursors of cholesterol. It is therefore important that any claim in experiments on the high yield of the conversion of mevalonic acid to cholesterol should be based on the radioactivity counts of the cholesterol after purification through the dibromo compound and not on the counts of the digitonides. It will be interesting to find out about the nature of the radioactive HCC in these experiments. As one should expect the fatty acids did not show any appreciable count in our experiment.

MILCH I note the presence of higher counting companions demonstrated in your experiments when samples of cholesterol are isolated within 2 hours after the administration of acetate C^{14}. You wouldn't expect, Dr Schwenk, if you had isolated the cholesterol through dibromide 24 hours after acetate administration that its count would differ from that of the total digitonide precipitable material.

SCHWENK We have not continued our experiment for more than 80 minutes mainly because of the small amount of mevalonic acid 2-C^{14} available. But from other experiments made with acetate 1-C^{14} we know that in experiments lasting much longer the HCC tends to decrease and after 1 week we find that all tissues including blood have practically the same low count in digitonide and dibromo cholesterol.

EDER In association with Drs L. I. Gidez and W. W. Shreeve (*Federation Proc 17 228 1958*) 56 μc of DL mevalonic acid 2-C^{14} was administered to a patient. Five per cent of this administered dose was found in the expired CO_2, which as Dr Popjak has pointed out corresponds very well to the labeled carbon removed in the conversion of lanosterol to cholesterol. About 35% of the administered C^{14} was found in the urine.

In Fig B is shown a curve for the incorporation of mevalonic acid into the plasma

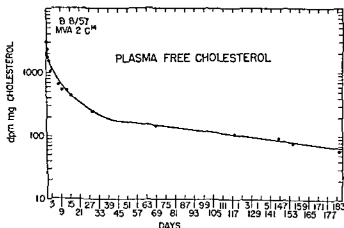


FIG B Curve for the incorporation of mevalonic acid into plasma free cholesterol

free cholesterol Appreciable incorporation occurred within 1 hour and maximal in corporation occurred at 4 hours The curve of incorporation was not a smooth curve perhaps this is due to the presence of high counting companions as suggested by Dr Schwenk After 12 hours a smooth disappearance curve was obtained At approximately 44 days the curve became linear and the slope remained constant throughout the period of observation The curve could be broken down into 4 straight lines these had half times of 0 42 2 7 9 5 and 99 days The curve with the very long half time suggests the presence of a very large cholesterol pool

POPJÁK I would like to make a few comments about the use of MVA *in vivo* for the possible study of derangements of cholesterol metabolism I think that it is not an ideal precursor for this purpose because it seems that the physiological con trol of the mechanism of cholesterol biosynthesis is somewhere between acetate and MVA formation Beyond MVA the synthesis goes with extraordinary speed Dr Gould when he visited us carried out some experiments along these lines Whereas the derangements of cholesterol metabolism can be readily demonstrated if we use C^{14} acetate as a precursor of cholesterol it is almost impossible to show a change when MVA is used as a precursor

GOULD I might add a comment to that there is one other difference between MVA and acetate incorporation into cholesterol *in vivo* For example in the rat acetate produces a higher specific activity of cholesterol in intestine than in liver on the other hand mevalonic acid is used hardly at all for synthesis in intestine and to a much less extent than acetate in other extrahepatic tissues suggesting that differences in permeability between liver and extrahepatic tissues to mevalonic acid may be influencing the results We have been unable to show any synthesis of cholesterol from MVA by intestine *in vitro* although acetate incorporation is readily demonstrable under the same conditions After MVA administration the labeled cholesterol will become distributed through the blood and will eventually reach more or less the same distribution in extrahepatic tissues as after acetate but the distribution is at first quite different

WHITE I was going to ask a general question in the area of hypercholesterolemia diabetes and atherosclerosis If the synthesis of cholesterol is dependent upon re duced TPN then one would expect in the diabetic an impaired synthesis of choles terol since the major source of reduced TPN in metabolism is the oxidation of glucose 6-phosphate to 6 phosphogluconic acid I am thinking of the work and postulates of Siperstein

EDER Siperstein showed that TPNH was required both for fatty acid and for cholesterol synthesis Apparently the requirement for TPNH is more explicit for fatty acid synthesis and so with the accumulation of acetoacetic acid cholesterol synthesis may occur even with a relative deficiency of TPNH

BLOCH It would appear that much less TPNH is required for cholesterol synthesis than for a molecule of a C16 or C18 fatty acid largely because the rate of fatty acid synthesis is so much faster at least normally Secondly the glucose 6 phosphate-dehydrogenase which produces TPNH for fatty acid synthesis is a com ponent of the soluble cytoplasm whereas the source of TPNH for steroid synthesis might be microsomal at least in part I think it could be explained on that basis

PINCUS Dr Popják maybe you would clarify this point

POPJÁK First of all I would like to support Dr Bloch in that there is much less TPNH required for sterol synthesis than for fatty acid synthesis and further more we do not know for certain in which of the reactions TPNH participates If it is right what you have said Dr Bloch that the reduction of the monocoenzyme

A ester of hydroxymethylglutaric acid to MVA requires TPNH then this is the only reaction in sterol synthesis in which it is known with any degree of certainty that TPNH is needed However TPN or TPNH seems to be required in squalene synthesis In our liver enzyme system it was very difficult to demonstrate whether TPNH or TPN was needed We had the impression from our experiments that the enzyme system worked more efficiently when both TPN and TPNH were present the yields of sterol were less if we either kept the TPN continuously reduced with excess glucose-6-phosphate or if we kept it continuously oxidized by the use of glutathione reductase and GSSG In the case of DPN the reduced form of the coenzyme is preferred by the system There is no absolute requirement by the liver enzyme system for either di or triphosphopyridine nucleotide but maximum activity was observed only when both were added to the incubations I think that reduced pyridine nucleotides whether DPNH or TPNH generated in either mitochondrial or microsomal systems could be—and probably are—readily transferred via the soluble parts of the cytoplasm from one to the other After all the DPNH and the TPNH (or their oxidized forms) that we are using in our enzyme system are introduced into the soluble component of the system and are presumably taken up by the microsomes In the diabetic TPNH could be generated by the isocitric dehydrogenase system

SMITH Only in mitochondria and not in microsomes

POPJÁK TPNH could be transferred into the microsomes

SMITH You cannot explain the failure of lipogenesis in the diabetic though

POPJÁK How about the transhydrogenase to form TPNH from DPNH?

SMITH Again that appears to be only in mitochondria

POPJÁK I give up although I believe pyridine nucleotides can be transferred from one cellular compartment to another

SMITH I think that is why Dr White raised the question The whole diabetic story seems so satisfying at the moment in terms of failure of phosphorylation of glucose whatever the reasons You have the failure of the oxidative pathway of glucose because this is the main supplier of TPNH and it is Dr Popjak who demonstrated that the reductive step of lipogenesis involves a TPNH specific reductase localized in microsomes Now on that basis one can explain the failure of lipogenesis in the diabetic whereas fatty acid degradation proceeds undiminished with the formation of ketone bodies Now if this is the case as I understand Dr White s question why is it that in the diabetic cholesterol synthesis occurs unimpaired even though this also requires TPNH or does the defect involve a failure to remove cholesterol rather than a failure in cholesterol biosynthesis?

MILCH Dr Popjak would you say that as indicated by the total degradation of cholesterol synthesized from C^{14} mevalonic acid localization of C^{14} atoms at five specific points is always demonstrated?

POPJÁK This follows from our results on squalene but the work on cholesterol itself is not ours That was done by Isler and his group at Hoffmann LaRoche in Basle and their results indicated that there are only five positions labeled They have isolated specifically position 7 position 22 and position 26 or 27—which cannot be distinguished These three positions were labeled No other position in the side chain was labeled and the specific activity of these isolated carbon atoms compared to the specific activity of the molecule as a whole indicated that there could be only five labeled positions in cholesterol formed from 2 C^{14} MVA

MILCH In contrast then in the biosynthesis of cholesterol from C^{14} acetate all the positions on the cholesterol molecule are available for label?

POPJAK Yes Certain positions are exclusively derived from the carboxyl carbon and other positions from the methyl carbon of acetate If you start with carboxyl labeled acetate as the precursor of cholesterol then you obtain two groups of carbon atoms in the sterol one group labeled and the other not labeled if you start with methyl labeled acetate then you get one group highly labeled and the other labeled slightly and those which are labeled slightly are the positions which come from the carboxyl carbon of acetate This slight mixing of labeling from methyl labeled acetate can be well explained by the formation of carboxyl labeled acetate from methyl labeled acetate via the citric acid cycle

MILCH It would seem then that there is an inconsistency in the reasoning that establishes mevalonic acid as a precursor of cholesterol *in vivo* in spite of the fact that the mevalonic acid was singly labeled in the 2 position

POPJAK No there is no inconsistency whatever

MILCH Of course the direct demonstration of such inconsistency would be accomplished by the biosynthesis of cholesterol from randomly labeled mevalonic acid Has such synthesis been done and has the resulting cholesterol been analyzed after total degradation?

POPJAK Well at the moment we are working with MVA 2 C^{14} with MVA labeled in carbon atom 4 and with MVA labeled both in the 3-methyl and in carbon atom 4 The degradation of cholesterol biosynthesized from these samples should give you the answer to your question but I don t think we have any doubt whatever that the labeling will be as can be predicted by our present data

GOULD Is MVA an intermediate in cholesterol biosynthesis in liver? In other words has anyone yet shown the formation of MVA from labeled acetate by liver homogenate or slices?

BLOCH According to substantiated rumors the accumulation of MVA has now been demonstrated in yeast

POPJAK Of course we might have expected that after all it seems that MVA is a by product of alcoholic fermentation We have seen repeatedly on paper chromatograms of hydroxamates made from liver enzyme systems a spot which is identical with the spot of hydroxamate of MVA but so far we have obtained only an ambiguous incorporation of acetate into that spot

SAMUELS I was interested in Dr Bloch s introduction of 6-hydroxylation in the transfer of the double bond The 6 hydroxylases are very prevalent in many tissues and affect steroids of shorter chains yet no explanation for their presence has been given I am wondering if you have observed whether the presence of these other compounds with shorter side chains would interfere with the conversion of lanosterol to cholesterol or whether you have ever done any trapping experiments with regard to this particular step

BLOCH The answer is no

WHITE I wonder if Dr Popjak would like to elaborate a little on the statement I believe he made that if there are regulators of cholesterol biogenesis these would be between acetate and mevalonate rather than beyond the latter

POPJAK Yes that is quite correct The evidence seems to point very definitely to the physiological control of sterol synthesis to be at the pre MVA stage It is possible to demonstrate some changes in cholesterol biosynthesis in different thyroid disorders if we use MVA as a precursor but these are rather slight in comparison with the changes that can be demonstrated with acetate as a precursor X irradiation causes a tremendous increase in the utilization of acetate for cholesterol synthesis yet very little increase in sterol synthesis can be demontrated if MVA is used as pre

cursor Also in cholesterol fed animals the utilization of acetate for cholesterol synthesis is suppressed almost to nil yet MVA is converted into cholesterol in these animals almost as efficiently as in the controls

SMITH Doesn t this almost limit the step to the condensation step between acetylacetyl CoA and acetyl CoA? There isn t really anything else We might as well pinpoint it

PINCUS The chairman would like to ask Dr Popják a question In describing the anaerobic production of squalene you in one of your slides showed a count of about 44 000 whereas when you demonstrated that steroid was also produced the total count was roughly half or about 22 000 Does this imply that squalene escapes into some other mechanism or is there something in the nature of the experiment which we missed as you described it?

POPJÁK No I think that there is something in the nature of the experiment that I missed too It was one of those anomalous things for which I cannot offer a sound explanation I think it is one of those experimental results that we meet from time to time The same enzyme preparation was used in both sets of experiments Generally I would say that in 90% of the experiments we had as good a synthesis of sterol as that of squalene In this particular experiment I don t exactly know why the sterol synthesis did not go so well

DICKMAN I would like to ask a question concerning the role of phosphate here Have either of you shown an absolute requirement for inorganic phosphate?

POPJÁK The high requirement for phosphate is a peculiarity of the liver enzyme system and its role is nothing else but the inhibition of phosphatases and thus the protection of the ATP For the isolation of intermediates formed from MVA in our earlier experiments we used barium precipitation and the presence of phosphate interfered in this process We tried to prepare therefore homogenates without a phosphate buffer We used KCl buffered with Tris for this purpose but the enzyme system was completely inactive we tracked down the troubl and showed that in the absence of phosphate the ATP was rapidly destroyed in the enzyme preparations The destruction of the ATP can be prevented by the use of either high concentrations of inorganic phosphate or by fluoride

CHAPTER 3

The Formation and Metabolism of Bile Acids under Different Conditions

SUNE BERGSTRÖM

Department of Physiological Chemistry University of Lund Lund Sweden[1]

To determine the over all metabolism of cholesterol one has to take into account (*a*) the cholesterol synthesis in the liver and in other organs (*b*) the cholesterol of the food and the extent of its absorption (*c*) the excretion of cholesterol and other neutral steroid derivatives (coprosterol etc) (*d*) steroid hormone production and excretion (*e*) bile acid formation and excretion with the feces

The analytical basis for making up the balance sheet is the fact that the steroid ring system is metabolically inert.

The rate of the different processes by which cholesterol is formed absorbed excreted and degraded are obviously of importance for the actual concentration of cholesterol in the blood

The synthesis (*a*) absorption (*b*) and excretion (*c*) will be dealt with by others at this symposium The amount of hormones formed (*d*) is of minor importance from a quantitative point of view

I will therefore first review some aspects of bile acid metabolism starting with the mechanism of formation and then turning to some quantitative aspects of these processes and the methods by which they can be determined

The main bile acid of the rat is cholic acid (80%) and most of the remainder is chenodeoxycholic acid with minor amounts of other metabolites all conjugated with taurine Some metabolic reactions are shown in Fig 1 The thick solid or interrupted lines show the metabolic reactions that have been demonstrated by administering labeled com pounds to rats The crossed thin lines show reactions that do not occur It is clear that chenodeoxycholic acid is not a precursor of cholic acid Two other trihydroxy acids are formed (11 and 12) as illustrated in Fig 2

The hydroxylations on the ring system have to take place before the side chain is oxidized otherwise no cholic acid is formed i e the 12α hydroxylase" does not work when the side chain is oxidized 3α 7α dihydroxycoprostane (3) yielded both chenodeoxycholic acid and cholic

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All pertinent references are available in a review (1) more recent papers are listed

acid but chenodeoxycholic acid or the corresponding C 28 acid did not yield any cholic acid The rate determining step might very well be the first hydroxylation at C 7 as all the hydroxylated compounds in the left column are transformed into bile acids much more rapidly than cholesterol itself The 7α hydroxylation of deoxycholic acid to cholic acid seems to be a peculiarity of the rat—it does not occur in rabbit or in man

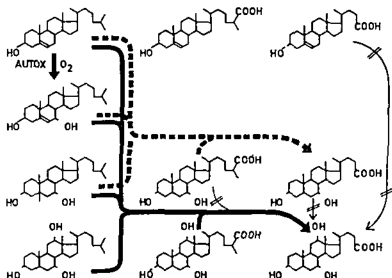


FIG 1 Some metabolic reactions

KEY Thick solid or interrupted lines metabolic reactions that have been demonstrated by administering labeled compounds Crossed thin lines reactions that do not occur

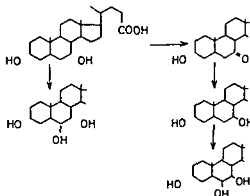


FIG 2 Formation of two trihydroxy acids in metabolism [Doisy *et al* (11 and 12) Bergstrom and Samuelsson (unpublished)]

This reaction has been studied with cholesterol stereospecifically labeled with tritium in position 7α or 7β (4) and it has been found that the 7α hydroxyl specifically replaces the 7α hydrogen leaving the 7β hydrogen intact

Normally taurocholate and taurochenodeoxycholate make up 90 to 95% of bile acids in rat bile and a chromatogram gives the simple curve shown in Fig 3 When the acids present in the feces of similar rats

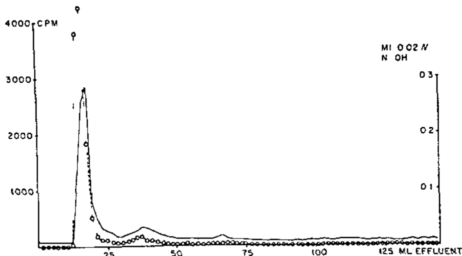


FIG 3 Chromatogram Rat 121 1 mg cholesterol-4 C^{14} I P Bile fistula made eighth day unhydrolyzed acids from ninth day MeOH 50 octanol 50 H_2O 50 chloroform 50 4 5 g hydrophic Supercel

are chromatographed a very complicated picture appears (Fig 4) Practically all conjugates have been split and a large number of metabolites appear Norman and Sjovall have recently concluded a study of the products formed (17) and the main results as to the cholic acid metabolites are summarized in Fig 5 One of the main reactions is the elimination of the hydroxyl group at 7 yielding deoxycholic acid subsequently oxidized to 12 ketolithocholic acid Another reaction is the dehydrogenation of the 7 hydroxyl to the 7 ketone that is then partially reduced again to the 7β isomer The two columns at the right in Fig 5 show a rough estimate of the proportions of the different acids in 2 rats of the same colony indicating the great variability to be expected of reactions caused by the intestinal flora The deoxycholic acid formed by the action of the intestinal flora is then rehydroxylated to cholic acid in the liver of the rat In recent experiments with doubly labeled cholic acid (7β T 24 C^{14}) (5) we have found that half of the

tritium of the cholic acid of the bile was lost after 24 hours When studying the turnover of the cholic acid pool in the rat this factor has to be taken into account

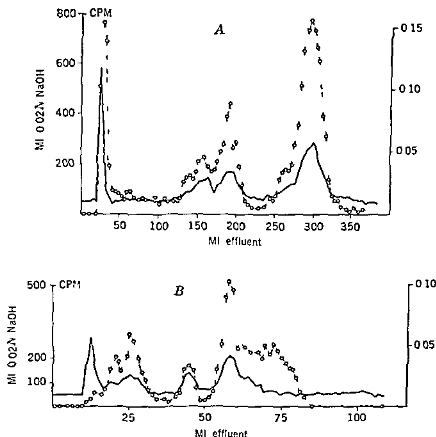


FIG 4 Chromatography of bile acids in feces of rat that has received cholesterol 4 C[14] (*A*) Rat 121 1 *mg cholesterol-4 C[14] I P unhydrolyzed fecal acids from* seventh day MeOH 50 octanol 50 chloroform 50 9 g hydrophic Supercel (*B*) Rat 121 1 mg cholesterol-4 C[14] I P less hydrophilic acid from unhydrolyzed fecal extract from seventh day MeOH 60 H O 40 chloroform 90 heptane 10 4 5 g hydrophic Supercel

Another striking example of the influence of the intestinal flora is found *in the rabbit* Rabbit *bile from the gall bladder contains prac*tically pure deoxycholic acid conjugated with glycine Lindstedt and Sjovall (15) found however that when you make a bile fistula in this animal the deoxycholic acid rapidly disappears and is replaced by cholic acid in the fistula bile Likewise if you give labeled cholic acid to an

intact rabbit labeled deoxycholic acid is formed in the gut—but it is not rehydroxylated to cholic acid in contradistinction to what happens in the rat Thus there seems to be an intestinal flora universally present in rabbits all around the world that causes what could have been thought to be the results of a special property of the rabbit liver cells Norman (unpublished results) has been able to reproduce these reactions *in vitro* with intestinal microrganisms

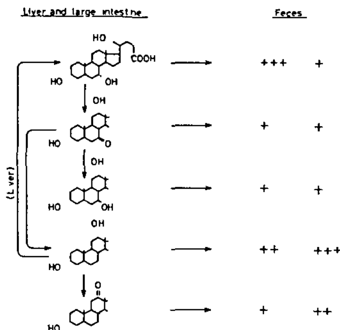


FIG 5 Main results as to the cholic acid metabolites summarized from a study recently concluded by Norman and Sjovall (17) The two columns at the right show a rough estimate of the proportions of the different acids in two rats of the same colony

A similar reaction occurs in man Deoxycholic acid present in the body seems to be entirely of microbiological origin in the gut and is not rehydroxylated in the liver (13) It is absent from bile of total fistulas that have functioned for a day or two (6) and also from the bile of new born babies (7) in which cases only cholic and chenodeoxycholic acids are present The intestinal flora in man is thus less efficient in transforming cholic acid into deoxycholic acid than that in the rabbit but both species are unable to rehydroxylate it to cholic acid in the liver

All these different reactions have to be taken into account, when the turnover rates and pool sizes are studied

In the rat you find generally a half life of about 2 to 3 days for any carbon labeled bile acid that is studied with a pool size of 20 to 25 mg in a 200 g rat it corresponds to a daily production of 5 to 6 mg bile acids per day However that is the production in the intact rat If a bile fistula is made the picture is entirely different An example from Eriksson's work (8) is shown in Fig 6 Most of the bile salts present

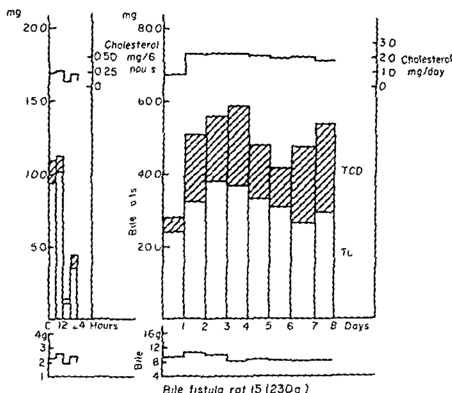


Fig 6 Excretion of bile acids (upper diagrams) and bile (lower diagrams) in bile fistula rat [Eriksson (8)] Values for the excretion of taurocholate (TC) and taurochenodeoxycholat (TCD) are expressed as mg/6 hr during the first day (left upper diagram) and as mg/24 hr during 8 days (right upper diagram)

in the rat have been excreted through the fistula after 2 hours (left part), but an increased synthesis results in the daily formation and excretion of 40 to 50 mg of bile acids per day or 10 times the normal daily synthesis In spite of the fact that about 3 times the amount of cholesterol present in the liver is transformed into bile acids daily and that there is a corresponding increase in the cholesterol synthesis in the liver no change in the blood cholesterol was found

Under these conditions the normal enterohepatic circulation is

broken and no bile acids reach the liver via the portal blood In recent simple experiments (2) we have shown that the continuous intestinal infusion of sodium taurochenodeoxycholate brought the cholic acid synthesis down into the normal region when 5 to 10 mg was injected

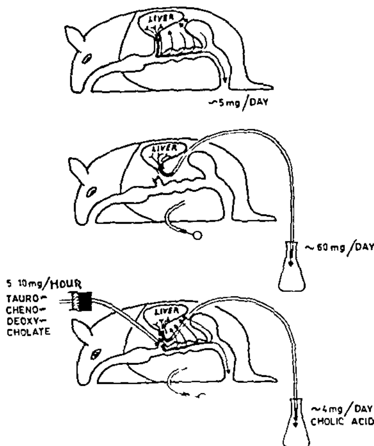


Fig 7 Schematic results exemplifying the continuous intestinal infusion of sodium taurochenodeoxycholate when injected into lower part of the bile duct of a fistula rat

into the lower part of the bile duct of a fistula rat These experiments are exemplified schematically in Fig 7

The actual concentration probably reaches only 2 to 3 mg % in the portal blood during these experiments but the bile acids are very efficiently absorbed so that the concentration in the general circulation is probably at most 0 1 mg % (Sjovall unpublished)

These results are also of interest in connection with the atherogenic diets containing free cholic acid that have been used in several studies. Generally the addition of cholic acid used has been 1 or 2%, i.e. if the rat eats 19 g. of food per day this corresponds to an average hourly intake of 5 to 10 mg. This addition can thus have a direct influence on the rate of bile acid formation. The free cholic acid in the diet is also conjugated and that results in a large demand on the organic sulfur compounds resulting in a more or less severe sulfur deficiency—another factor of importance for producing atherosclerosis in experimental animals. In order to study the influence of bile acids per se they should be added to the diet as taurine conjugates.

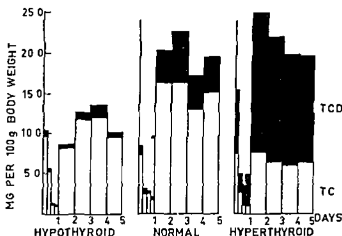


FIG. 8. Bile acid excretion in hypo-, eu-, and hyperthyroid bile fistula rats—average of 4 animals in each group (Eriksson).

There are quite pronounced changes in the bile acid picture in the hyper- and hypothyroid state. Some data published by Eriksson (9) are shown in Fig. 8. In the normal rats there is the usual mixture of about 80% cholic acid and 20% chenodeoxycholic acid. In the hypothyroid rats there is almost only cholic acid present, whereas in the hyperthyroid state there is more chenodeoxy- than cholic acid. A possible explanation is obviously that the side chain degradation is speeded up relative to the 12-hydroxylation in the latter case, whereas the reverse is true in the former case. The results illustrate anyhow the necessity of analyzing the different bile acids present in order to get a quantitative picture—if only the cholic acid had been followed in this example the results would have been quite misleading.

Another difference between these groups is that the normal and hypothyroid rats excrete 1 to 2 mg. of cholesterol in the bile fistula per

day whereas the hyperthyroid rats excrete about 6 mg as has earlier been observed by Rosenman *et al* (18)

If we now again turn to the quantitative aspects of bile acid metabolism in normal rats it has been found by Lindstedt and Norman that the half life of the different common bile acids was about 2 days The technique is simply to give a tracer dose of carbon labeled bile acid *per os* and follow the rate of excretion of the isotope with the feces

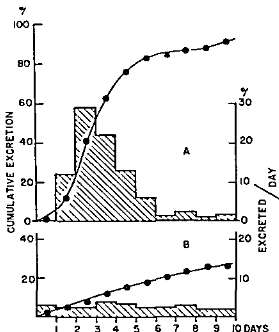


FIG 9 Excretion of labeled material in feces after intraperitoneal injection of cholic acid 24 C^{14} into a normal rat (*A*) and one treated with chemotherapeutics (*B*)

They also found that the rate was very markedly decreased with half life times of 10 days if the rats were treated with antibiotics at the same time (Fig 9)

To study this question further we are collaborating with Dr Bengt Gustafsson at Lund in studying various aspects of the steroid and bile acid metabolism in the germ free rats of the colony that he has reared at Lund These animals appear normal in all respects except that there is a marked distention of the cecum

The four experiments shown in Figs 10 and 11 (10) have been performed on the same germ free rat and the data are recorded in the usual semi logarithmic plot (Fig 10) In the germ free condition as well as

after infection with *Aspergillus niger* and *Clostridium perfringens* there is no significant change in the rate of excretion After total infection there was however an immediate return to the normal half life

The chromatograms of the labeled compounds present in the feces of that rat during these different periods are shown in Fig 11 Under germ free conditions and after infection with *Aspergillus* the chromatograms are identical with the picture in the bile i e no changes take

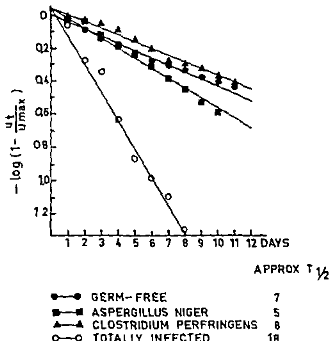


Fig 10 Semilogarithmic plot of the excretion of labeled material in a rat during periods with different conditions in the intestine [Gustafsson et al 1957 (10)]

place during the passage through the intestinal tract When *Clostridium* was present the taurine conjugates had been split to a large extent and the main labeled compound present was free cholic acid After total infection most of the conjugate had been split but furthermore the cholic acid had been transferred into a number of other metabolites

The intestinal flora has thus a pronounced influence and possible changes of the intestinal flora must also be considered when dietary changes are made

In these connections I would like to remind you of the interesting work of Dam and his colleagues on the dietary production of cholesterol gallstones in hamsters another demonstration of the profound influence of the diet on the cholesterol metabolism

In man the main bile acids are cholic chenodeoxycholic and deoxycholic acid that undergo secondary changes to varying degrees in the intestine In order to determine the total amount present and its turnover Lindstedt (14) has used the following approach

A tracer dose of carbon 14 labeled cholic acid was administered by mouth Subsequently every second or third day small samples of

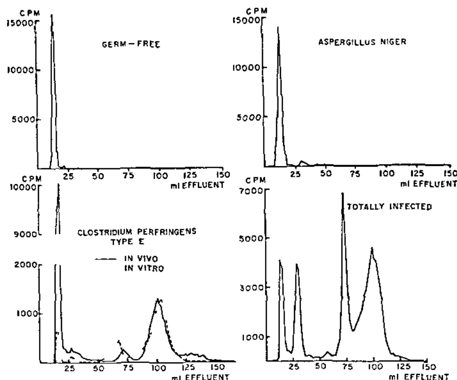


FIG 11 Chromatography of fecal acids excreted during periods with different conditions in the intestine (Gustafsson Bergstrom Lindstedt and Norman 1956)

pure bile are collected via a thin plastic duodenal tube When the tube has reached the duodenum 1 mg of cholecystokinin (obtained from Professor E Jorpes Stockholm) is given intravenously resulting in an immediate contraction of the gall bladder The small sample of bile that was taken up is hydrolyzed and pure cholic acid isolated, and its specific activity determined The relative amounts of the other bile acids are determined at the same time with the aid of Sjovall s quantitative methods

By following the decrease in the *specific activity of the cholic acid*

with time and extrapolating back to zero time an estimate of the cholic acid pool and its turnover is obtained (Fig 12)

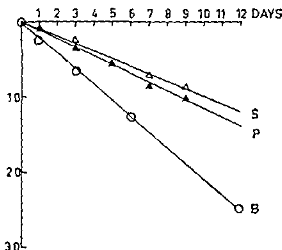


Fig 12 Semilogarithmic plot of the fall of specific activity of cholic acid in human bile [Lindstedt 1957 (14)]

The data from 6 male students are shown in Table I The daily synthesis is fairly constant about 1 g It is apparent that the smaller the pool the more rapid the turnover

TABLE I
Bile Acid Production in Man

Subject	Half life (days)	Circulating pool: Cholic acid (g)	Circulating pool: Total bile acids (g)	Daily formation: Cholic acid (g)	Daily formation: Total bile acids (g)
S	3	1 29	3 5	0 30	0 8
P	2 5	0 92	3 6	0 25	1 0
B	1 2	0 58	1 8	0 33	1 1
W	2 3	1 15	3 3	0 35	1 0
E	2 7	2 29	4 8	0 59	1 2
A	3	1 18	3 2	0 27	0 8

ᵃ Lindstedt [1957 (14)]

These studies are now being extended to different pathological cases If the cholesterol present in the liver and the blood is estimated to 12 to 15 g these results check reasonably well with the half life of 8 days found by London and Rittenberg (16)

However there are still no exact data available of the amount of endogenous steroids excreted on a normal triglyceride-containing diet entirely free of steroids

In Fig 13 there is finally a summary of the turnover of cholesterol in man The cholesterol coming in with the food is generally not more than 1 g and usually much less However with the bile at least 1 g and apparently often about 2 g of cholesterol is secreted into the intestine apart from the steroids following other intestinal juices and shed cells Most of the former cholesterol is however reabsorbed It is thus apparent that the cholesterol of the diet is less than the endogenous cholesterol introduced into the intestine Furthermore about 1 g of cholesterol is degraded to bile acids per day

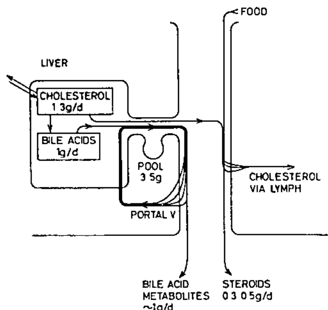


FIG 13 Summary of the turnover of cholesterol in man

Clearly the process of intestinal absorption is of prime importance for cholesterol metabolism—an impairment of the absorption can rapidly lead to loss of considerable amounts of cholesterol The question is then to what extent and how rapidly this loss stimulates increased endogenous synthesis

REFERENCES

1 Bergstrom S and Borgstrom B *Ann Rev Biochem* **25** 177 (1956)
2 Bergstrom S and Danielsson H *Acta Physiol Scand* **43** 1 (1958)
3 Bergstrom S and Lindstedt S *Biochim et Biophys Acta* **19** 556 (1956)
4 Bergstrom S Lindstedt S Samuelsson B Corey E J and Gregoriou G *J Am Chem Soc* **80** 2337 (1958)
5 Bergstrom S Lindstedt S and Samuelsson B *Acta Chem Scand* in press

with time and extrapolating back to zero time an estimate of the cholic acid pool and its turnover is obtained (Fig. 12).

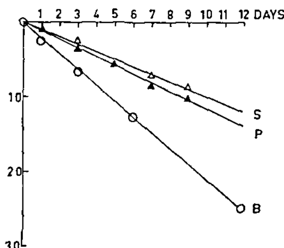


Fig. 12. Semilogarithmic plot of the fall of specific activity of cholic acid in human bile [Lindstedt 1957 (14)].

The data from 6 male students are shown in Table I. The daily synthesis is fairly constant, about 1 g. It is apparent that the smaller the pool the more rapid the turnover.

TABLE I
Bile Acid Production in Man

Subject	Half life (days)	Circulating pool: Cholic acid (g)	Circulating pool: Total bile acids (g)	Daily formation: Cholic acid (g)	Daily formation: Total bile acids (g)
S	3	1.29	3.5	0.30	0.8
P	2.5	0.92	3.6	0.25	1.0
B	1.2	0.58	1.8	0.33	1.1
W	2.3	1.15	3.3	0.35	1.0
E	2.7	2.29	4.6	0.59	1.2
A	3	1.18	3.2	0.27	0.8

[a] Lindstedt [1957 (14)].

These studies are now being extended to different pathological cases. If the cholesterol present in the liver and the blood is estimated to 12 to 15 g, these results check reasonably well with the half life of 8 days found by London and Rittenberg (16).

However, there are still no exact data available of the amount of endogenous steroids excreted on a normal triglyceride containing diet entirely free of steroids.

body going via the blood to the liver and the bile and then you have another source of fecal steroids directly from the wall of the intestinal tract

WHITE You feel then that while there may be cholesterol turnover in other tissues there probably is very little cholesterol degradation?

BERGSTROM Well nobody knows because it might be that the first step the hydroxylation takes place in other tissues We know that hydroxy cholesterol has been isolated from blood but nobody knows if it was an autooxidation product or if it was present originally in the blood

KENDALL There are two questions that have been suggested to me One the possibility of the synthesis of bile acids by tissues other than liver Chaikoff has shown that tissues other than liver will yield labeled CO_2 when incubated with C-26-labeled cholesterol This suggests that bile acids are being formed in these tissues as well as in the liver Is the liver the only organ that is actively synthesizing bile acids or do all tissues slowly break down cholesterol to give bile acids which would be concentrated in the liver and excreted in the bile? The second possibility I wanted to raise was whether all of the bile acids are formed by the oxidation of cholesterol or whether some of the bile acids might possibly be synthesized directly from acetate One observation that we made has perhaps bearing on both points Rats in which bile fistulas had been established 3 days previously were given a single intraperitoneal injection of labeled acetate and the cholesterol and bile acids in the fistula bile were isolated and their radioactivity determined each day for the ensuing 2 weeks Now in the first samples of bile collected after the injection of the acetate the bile acids had an activity considerably greater than the activity of the cholesterol being excreted in the bile but after the first 12 hours and for the remaining 14 days of the experiment the specific activity of the bile acids was consistently 75% of the activity of the cholesterol being excreted at the same time The specific activity of the bile acids and the cholesterol decreased at a parallel rate with a half life time of about 5 days for each The amount of labeled bile acid recovered amounted to about 50 mg per day At the end of the period the activity of the cholesterol in the liver and the blood was the same as the activity of the cholesterol in the fistula bile In these 14 days the rat was excreting labeled bile acid at the rate of 50 mg per day with a turnover time of about 7 days That would require about 300 to 350 mg of a labeled pool of cholesterol which approximates pretty closely the total amount of cholesterol in the carcass of a rat exclusive of perhaps the brain and spinal cord It looked as though the total carcass cholesterol in the bile fistula rats was being involved in the synthesis of bile acids And the lower specific activity of the bile acids might indicate that bile acid was continuously synthesized from unlabeled precursors as well as from labeled cholesterol

BERGSTROM First to the information on oxidation of cholesterol in other tissues I think that is a matter of degree really I think most tissues have a small capacity *to oxidize methyl groups and to beta oxidize—it is a general property* Any hydrocarbon or a steroid of any configuration which you inject behind the wall of the gut is oxidized to some kind of acid so I think it quite possible that other tissues might form some kind of acid but I doubt very much that they are the regular ones of the animal because as soon as any step is not in the right order everything goes astray If you give cholanic acid you get a mixture of many different compounds formed by hydroxylation in many different ways The exact amount nobody knows but I don t think it is of any great quantitative importance

KENDALL In view of the greater mass of peripheral tissues as compared to the

6 Ekdahl P H and Sjovall J Acta Chir Scand 114 439 (1958)
7 Enerantz J C and Sjovall J Acta Chem Scand 11 1092 (1957)
8 Eriksson S Proc Soc Exptl Biol Med 94 578 (1957)
9 Eriksson S Proc Soc Exptl Biol Med 94 582 (1957)
10 Gustafsson B E Bergstrom S Lindstedt S and Norman A Proc Soc Exptl Biol Med 94 467 (1957)
11 Hsia S L Matschiner J T Mahowald T A Elliott W H Doisy E A Jr Thayer S A and Doisy E A J Biol Chem 226 667 (1957)
12 Hsia S L Matschiner J T Mahowald T A Elliott W H Doisy E A Jr Thayer S A and Doisy E A J Biol Chem 230 573 (1958)
13 Lindstedt S Arkiv Kemi 11 145 (1957)
14 Lindstedt S Acta Physiol Scand 40 1 (1957)
15 Lindstedt S and Sjovall J Acta Chem Scand 11 421 (1957)
16 London J M and Rittenberg D J Biol Chem 184 687 (1950)
17 Norman A and Sjovall J Biochim et Biophys Acta in press (1958) J Biol Chem in press (1958)
18 Rosenman R H Byers S O and Friedman, M J Clin Endocrinol and Metabolism 12 1287 (1952)

Discussion

WILHELMI May I start by asking Dr Bergstrom how he concluded that the characteristic bile acids of the rat the rabbit and the pig are truly international?

BERGSTROM We have not much experience with the pig In the rabbit every body has reported deoxycholic acid as the main bile acid—there has been only one report by a Japanese author some 20 years ago that his rabbits had deoxycholic acid during winter and cholic acid during summer or vice versa This might possibly be due to a changed intestinal flora due to a drastically changed diet

WHITE My question concerns the problem of total cholesterol balance and possible pathways of metabolism other than bile intestine feces I think that the data that are available suggest that certainly the major metabolic pathway of cholesterol is transformation to bile acids and excretion via the bile Are there other tissues in the body which can degrade cholesterol and what is known about the proportion of cholesterol that is metabolized by other tissues? Is there cholesterol degradation for example by some of the larger blood vessels? In addition we know that there is some cholesterol degradation although perhaps relatively small in some endocrine glands What is known about the capacity of other tissues to metabolize cholesterol in terms of cholesterol balance since I think what is accounted for in your balance sheet as I calculate it represents a very small fraction of the total cholesterol in the circulating blood for example?

BERGSTROM Say you have 15 g in the liver blood pool in man

WHITE Well I came out with 1.5 Let us assume 15 g in the blood You have allowed for approximately 1.5 g via bile acids What is known about the fate of cholesterol in the blood which is not excreted by the enterohepatic mechanism via bile into feces?

BERGSTROM First of all if you give ring labeled cholesterol it seems that the specific activity of the different cholesterol pools in 24 hours is rather evenly labeled but nobody has really looked carefully You get about 80 to 90% of the cholesterol as bile acids in the feces during the next 2 to 3 weeks and 10 to 20% as steroids but then in the feces you have more steroids than that corresponding to the 10% and that cholesterol is I think from the gut You have one cholesterol pool in the

We feel as Dr Bergstrom has pointed out that this occurs because the newly formed biosynthetic cholesterol in the liver is initially diluted only with the liver cholesterol pool which is smaller than the circulatory cholesterol pool which subsequently acts as a diluent

I would like to make a comment concerning pool sizes and turnover rates A turnover rate is a function of the length of time over which it is observed If a process is followed for 10 days one observes a different turnover time from that observed if the same process is followed for 30 days As the disappearance curve of cholesterol is followed the rate of disappearance continuously slows down It is extremely difficult by any means of analysis available at present to assign a unique half life time or turnover time to cholesterol If a standardized length of observation is adopted perhaps it may be possible to assign reproducible values to the turnover time With respect to pool size the values quoted by Dr White and Dr Bergstrom make use of the liver and circulating cholesterol which together account for perhaps 15 g This pool represents a minimum size and the actual pool may be larger If you consider that as Dr Bergstrom says 500 mg of sterol are excreted in the feces along with a gram of bile acids this amounts to a daily excretion of 1 5 g of steroid At the minimum pool size of 15 g this yields a turnover time of 10 days If the pool size were closer to 30 g this would yield a turnover time of 20 days a value commonly reported for cholesterol Therefore I do not think there is any necessity for invoking other mechanisms to account for the disposition of cholesterol I think that the data are internally consistent as they have been presented

Bloch I want to ask Dr Bergstrom whether when he talks about cholesterol excretion he means cholesterol only or would this include coprosterol? It seems to me that coprosterol is rarely mentioned in connection with sterol balances and it must occur in very substantial amounts in the excreta of at least some animal species

Bergstrom I started out talking about cholesterol and its congeners We have not cleared that up yet but in the germ free animals there seem to be small amounts of other steroids besides cholesterol

liver a slow process there might result in the production of an appreciable part of the bile acid formed by the intact animal

BERGSTROM Has anybody examined this in hepatectomized animals?

KENDALL In that case you would be dealing with a short term experiment, and it is doubtful that the slow production of bile acid could be demonstrated in the period in which the animal would remain alive

BERGSTROM Well we can get some indication when Dr Sjovall has cleared up the quantitative determination of the bile acids from blood There is no method as yet to determine that I think your data on the half life corroborates our work on bile fistula rats If you give a dose of tracer cholesterol to normal rats it is excreted with a half life of maybe 25 days In the fistula rat we find a half life of 6 days That checks very nicely with yours Don t you think your data might just as well be explained by some of the newly formed cholesterol being directly oxidized to bile acid? It is all microsomal reactions

KENDALL Wouldn t you expect the cholesterol being excreted by the liver to be diluted with the freshly synthesized cholesterol as much as the bile acids being diluted by the oxidation of the freshly synthesized cholesterol?

BERGSTROM In the liver cell you have a continuous cholesterol synthesis from acetate There is further a continuous excretion and uptake from the blood of cholesterol In a bile fistula there is 10 times faster cholesterol synthesis than normal—3 times the actual amount of cholesterol present in the liver is transformed into bile acids daily After a dose of labeled acetate it seems likely that some of the newly synthesized cholesterol might be shunted into the bile acid formation before complete mixing with the liver blood pool Later on when the newly synthesized cholesterol is unlabeled and the labeled cholesterol is circulating the reverse might occur i e the bile acids are less active than the cholesterol pool

KENDALL Of course both hypotheses would explain the high activities of the first bile acid to be excreted after the injection of acetate

BERGSTROM How much stronger was it?

KENDALL About 50% stronger

BERGSTROM Then it went down to about 75% ?

KENDALL Yes

BERGSTROM Well that checks fairly well In the first place the newly synthesized cholesterol partially goes directly later the newly synthesized unlabeled cholesterol is diluting the labeled cholesterol coming back from the blood

KENDALL Can you see any way of experimentally differentiating between these two possibilities?

BERGSTROM We had to determine the rate by which cholesterol is coming in from the blood at the same time If we could fix that we could account for it I guess You need to determine the specific activity and the amount of cholesterol coming in from the blood versus the cholesterol and the bile acid in the bile

KENDALL If this was studied in cholesterol fed animals where the hepatic synthesis of cholesterol is reduced would that be a valid approach? Do you see what I mean?

BERGSTROM Yes quite But I doubt that you can depress it totally in a bile fistula animal

KENDALL Yes

HELLMAN We have some data bearing on this point In patients with bile fistulae the initial sample of biliary cholesterol after feeding labeled acetate is uniformly of higher specific activity than subsequent samples of cholesterol in the bile

We feel as Dr Bergstrom has pointed out that this occurs because the newly formed biosynthetic cholesterol in the liver is initially diluted only with the liver cholesterol pool which is smaller than the circulatory cholesterol pool which subsequently acts as a diluent

I would like to make a comment concerning pool sizes and turnover rates A turnover rate is a function of the length of time over which it is observed If a process is followed for 10 days one observes a different turnover time from that observed if the same process is followed for 30 days As the disappearance curve of cholesterol is followed the rate of disappearance continuously slows down It is extremely difficult by any means of analysis available at present to assign a unique half life time or turnover time to cholesterol If a standardized length of observation is adopted perhaps it may be possible to assign reproducible values to the turnover time With respect to pool size the values quoted by Dr White and Dr Bergstrom make use of the liver and circulating cholesterol which together account for perhaps 15 g This pool represents a minimum size and the actual pool may be larger If you consider that as Dr Bergstrom says 500 mg of sterol are excreted in the feces along with a gram of bile acids this amounts to a daily excretion of 1 5 g of steroid At the minimum pool size of 15 g this yields a turnover time of 10 days If the pool size were closer to 30 g this would yield a turnover time of 20 days a value commonly reported for cholesterol Therefore I do not think there is any necessity for invoking other mechanisms to account for the disposition of cholesterol I think that the data are internally consistent as they have been presented

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Chapter 4

Thyroid Function Thyroxine Analogs, and Cholesterol Metabolism in Rats and Rabbits

G S Boyd

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Introduction

If a substance could be found which would lower the serum cholesterol level and yet be free from undesirable side effects then the way would be open to test the validity of the concept that it is desirable to lower the serum cholesterol levels of countless humans

It is well known that the activity of the thyroid can influence the circulating cholesterol level in the human (7) The administration of thyroid extract however elevates the basal metabolic rate as well as depressing the plasma cholesterol level The principal compounds secreted by the thyroid are thyroxine and triiodothyronine and these iodinated amino acids appear to be modified by various tissues through such reactions as partial deiodination oxidative deamination decarboxylation etc Consequently from the parent tetraiodinated substance—thyroxine—a vast number of metabolites are theoretically possible and many have already been isolated from tissues or synthesized (4)

The question then arises as to whether it is possible to dissociate the plasma lipid depressant and the metabolic stimulant effects of these thyroxine analogs and this involves a search for a derivative active in the former and inactive in the latter respect Some of the laboratory data which we have collected on the thyroid and a small group of substances related to thyroxine are presented in this paper

Material and Methods

The animals used in this study were inbred Wistar strain albino rats reared on stock rat cake similar to diet 41 (3) The rabbits employed were an inbred Chinchilla strain also reared on a stock rabbit diet

Oxygen consumption rate measurements were performed by the technique and apparatus described by D Amour and Blood (6) or that of Tomich and Woollett (11) and the results are expressed in liters of oxygen per kg body weight per hour

Blood samples (about 0 3 ml) were obtained by cutting the tip of the rat s tail and the blood was allowed to clot at room temperature

Serum cholesterol determinations were performed by a micromodification of the Sperry and Webb (10) procedure to allow duplicate estimations of this sterol from 0 10 ml serum

Relative rates of hepatic cholesterol synthesis were obtained by incubating 1 g liver slices in 10 ml Krebs Henseleit Ringer containing $CH_3 C^{14}OO^-Na^+$ (acetate C^{14}) The incubations were performed under 100% oxygen for 3 hours at 37 C shaking at 110 oscillations per minute After incubation the tissues were saponified by alcoholic potassium hydroxide and the sterol was then extracted with petroleum ether This extract was washed with water 1% sodium acetate solution water washed again and dried over anhydrous sodium sulfate and taken to dryness The cholesterol was precipitated as cholesterol digitonide and a weighed quantity combusted to CO which was trapped by Ba(OH) and the radioactivity of the precipitated BaCO was then counted at infinite thickness

Tissue oxygen consumptions were determined by the conventional Warburg technique The tissues were sliced by hand the incubating medium was Krebs Ringer glucose the gas phase 100% O and measurements were made over 30 to 40 minutes at 37 C

Hypothyroidism in the Rat

There are three well recognized methods of suppressing thyroid function in mammals namely surgical excision of the gland destruction by radioactive iodide and the oral administration of antithyroid drugs such as thiouracil It seems of interest to contrast the effect on cholesterol metabolism of hypothyroid states induced by these different methods

1 *Thyroidectomy*

Young adult male rats (150 g) were thyroidectomized under ether by the usual method control" animals were "sham operated Post operatively the animals were allowed a calcium lactate solution to drink and the normal stock rat cubes to eat *ad lib* The serum cholesterol of these animals was studied before operation and for 10 weeks post operatively It was found that the mean preoperative serum cholesterol level was 75 $\pm$ 8 mg % whereas postoperatively the level rose to 87 $\pm$ 7 mg % This slight rise in serum cholesterol after thyroidectomy contrasts sharply with the highly significant drop in oxygen consumption rate exhibited by these animals This fell from a preoperative level of 2 1 liters/kg /hour to 1 44 liters/kg /hour at the sixth week after operation The rate of hepatic cholesterol biosynthesis of the thyroidectomized rats was only 69% of the corresponding biosynthesis rate for the sham

operated control animals and the tissue oxygen consumption of the thyroidectomized rats was markedly depressed (see Table I)

TABLE I

THE EFFECT OF THYROIDECTOMY RADIOACTIVE IODINE AND THIOURACIL ON THE SERUM CHOLESTEROL HEPATIC CHOLESTEROL BIOSYNTHESIS AND OXYGEN CONSUMPTION OF ADULT MALE RATS

Method	Serum cholesterol (mg %)	Oxygen consumption rate (l/kg/hr)	Relative rate of cholesterol synthesis	Percentage change in liver QO
Control	75 ± 8	2 1	100	100
Thyroidectomy	87 ± 7	1 44	69	79
I^{131} Iodine	89 ± 7	1 35	57	68
Thiouracil	109 ± 9	1 45	67	77

2 Thyroid Destruction by I^{131}

Male rats weighing about 150 g were injected intraperitoneally with 900 μc radioactive iodine The oxygen consumption rate and the serum cholesterol were determined before and after treatment with radioactive iodine It was found that while the oxygen consumption rate of the I^{131} treated animals was significantly depressed the serum cholesterol level was not significantly elevated The tissue oxygen consumption and the hepatic cholesterol biosynthesis were determined on killing the animals and it was found that both the QO and the cholesterol bio synthesis were markedly depressed (see Table I) The tracheas of these animals were serially sectioned and there was no evidence of functional thyroid tissue

3 Thyroid Suppression by Thiouracil

Young adult male rats were fed the stock diet to which 0 3% thiouracil had been added The oxygen consumption rate and serum cholesterol levels were recorded on these animals over a period of about 12 weeks It was found that the serum cholesterol levels rose quite quickly to a value about 25% higher than the control level while the basal metabolic rate dropped within about 3 weeks to a value of approximately 30% below the control level (see Fig 1) On killing these animals the tissue oxygen consumption and liver cholesterol biosynthesis were measured as before and it was found that the values obtained were significantly lower than the corresponding control values for animals maintained on a diet free of thiouracil (see Table I) Thus in our strain of rats total thyroidectomy and I^{131} ablation of the gland produced comparable effects on the serum cholesterol level These results were distinct from the effects of dietary 0 3% thiouracil induced hypothyroidism

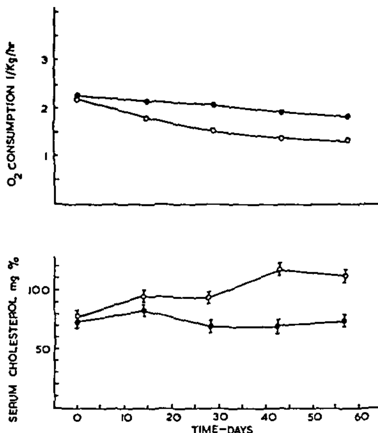


FIG 1 The effect of thiouracil on the oxygen consumption and serum cholesterol of adult male rats

THE EFFECT OF RADIOACTIVE IODINE AND THIOURACIL ON THE OXYGEN CONSUMPTION RATE AND SERUM CHOLESTEROL OF YOUNG MALE RATS

In this study it was decided to investigate whether thiouracil exerted an influence on the cholesterol metabolism of the athyreotic rat Sixteen young male rats were divided into two groups of 8 rats per group One group was injected intraperitoneally with 600 μc I^{131}/100 g body weight and the oxygen consumption and serum cholesterol levels of all the animals were recorded at regular intervals for about 6 weeks Each group was then subdivided into two equal groups one of which drank 5% glucose and the other 5% glucose containing 0 1% thiouracil This regimen was continued for a further 6 week period during which the oxygen consumption and serum cholesterol were recorded (see Fig 2) The animals were killed and the QO measured as described above The thyroids or thyroid "remnants" were examined histologically

The results of this experiment are shown in Figs 2 and 3 from which it can be seen that the inclusion of 0 1% thiouracil in the drinking water of these rats while exerting a pronounced effect on the oxygen consumption of the rats with intact thyroids failed to influence the already depressed oxygen consumption of the animals which had been subjected

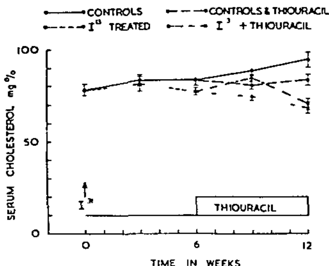


Fig 2 The effect of I^{131} and thiouracil on the serum cholesterol of rats

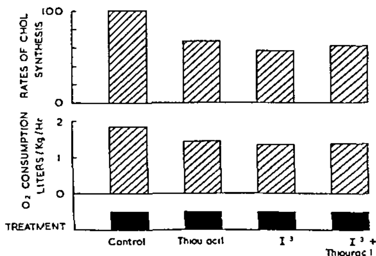


Fig 3 The effect of I^{131} and thiouracil on the oxygen consumption and rate of cholesterol synthesis in the rat

to thyroid ablation by radioactive iodine Furthermore the serum cholesterol levels of these groups were not significantly different Thus the level of thiouracil in the diet appears to be of importance in dissociating the goitrogenic dose from the hypercholesterolemic dose

The Effect of Dietary Thiouracil on the Serum Cholesterol of Athyreotic Cholesterol Fed Rats

Young male rats were thyroidectomized and treated as described previously Their oxygen consumption rate was recorded postoperatively and any animal failing to show at least a 20% drop in oxygen consumption was discarded Six weeks after operation they were divided into two groups One was fed the stock diet containing 1 0% cholesterol plus 0 3% thiouracil while the other was fed the stock diet containing 1 0% cholesterol After 4 weeks, the serum cholesterol levels of the groups were studied and it was found that the athyreotic thiouracil treated group had serum cholesterol levels of 131 ± 14 while the athyreotic (controls) had serum cholesterol levels of 98 ± 9 (see Table II) Furthermore the concentration of cholesterol in the livers of the thiouracil treated animals was 50% higher than in the "control group Thus thiouracil had not impaired cholesterol absorption

TABLE II

The Effect of Dietary Thiouracil on the Serum Cholesterol of Athyreotic Cholesterol Fed Rats

Group	Number of animals	Diet	Oxygen consumption (l /kg /hr)	Serum cholesterol (mg %)
Thyroidectomized	8	1% Cholesterol + 0 3% thiouracil	1 39	131 ± 14
Thyroidectomized	8	1% Cholesterol	1 55	98 ± 9

The evidence accumulated by these experiments appears to suggest that in our rat colony thiouracil influences cholesterol metabolism not only through its anti thyroid action which depresses cholesterol synthesis but also by some other mechanism This could be on cholesterol oxidation excretion or modification of the intestinal flora Despite intensive study the mode of action of thiouracil as an anti thyroid compound has not been settled unequivocally One theory is that thiouracil exerts its anti thyroid effect through its anti oxidant properties The possibility must therefore be considered that the accumulation of cholesterol in animals fed thiouracil plus cholesterol is due to inhibition of certain steroidal catabolic oxidative reactions

The Effect of Thyroxine and Thyroxine Analogs on Cholesterol Metabolism and Tissue Respiration in the Rat

In this study it was decided to investigate the effects of thyroxine triiodothyronine tetrac and triac on the serum cholesterol the basal metabolic rate the hepatic cholesterol biosynthesis and the oxygen consumption of various tissues obtained from rats treated with these substances The animals used in this study were male adult rats which were allowed access to food *ad lib* The thyroactive compounds were administered subcutaneously daily in 0 005 N sodium hydroxide solution The oxygen consumption rates of the animals were recorded daily and the doses of substances employed were such that the animals in different groups exhibited somewhat comparable rises in oxygen consumption

After 5 days treatment with these substances the animals were killed and the observations shown in Fig 4 were made The results of this study showed that the administration of thyroxine and thyroxine analogs to euthyroid rats while producing an elevation in the basal metabolic rate resulted in no concomitant decrease in the serum cholesterol as is observed in the euthyroid human under these circumstances The serum cholesterol levels of the treated animals were slightly higher than those prevailing in the control animals The rates of hepatic cholesterol biosynthesis roughly paralleled the rise in basal metabolic rate so that in all the hyperthyroid animals the rate of cholesterol synthesis was elevated The oxygen consumption of excised tissues from these animals showed a very marked differential effect on the respective QO It was found that heart tissue responded more rapidly and quantitatively to a greater degree than any other tissue examined while kidney exhibited a less marked elevation in oxygen consumption and liver showed the lowest response of the 3 tissues examined

These results confirm our earlier observations with triac (2) and are in agreement with the *in vitro* tissue respiration observations of Barker (1) Thus in the normocholesterolemic euthyroid rat the subcutaneous administration of these thyroxine analogs raised the oxygen consumption but failed to influence the serum cholesterol level By contrast Cuthbertson *et al* (5) have shown that in hypercholesterolemic rats triiodothyronine exhibits a cholesterol depressant effect The thyroxine analogs produce a marked differential effect on tissue oxygen requirements and of all the tissues examined cardiac tissue was found to be the most sensitive This effect of thyroxine analogs on cardiac respiration is further complicated by the marked hypertrophy of the heart which presumably accompanies the increased cardiac output so that after some time although the total oxygen requirement of the heart is elevated the calculated QO is decreased

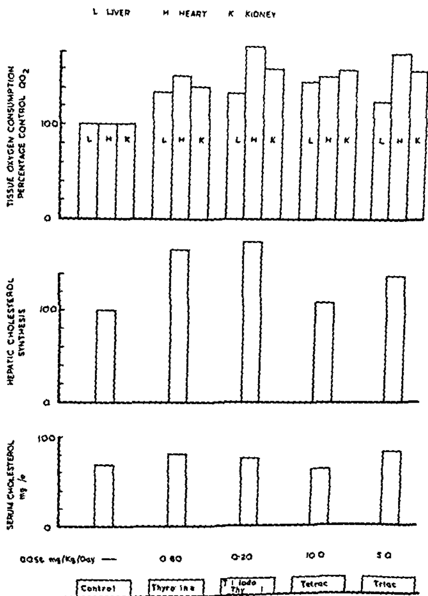


Fig 4 The effect of thyroxine and thyroxine analogs on cholesterol metabolism and tissue respiration in the rat

THE EFFECT OF TRIAC ON EXPERIMENTAL ATHEROMA IN THE RABBIT

Aortic atheromatous lesions are induced in rabbits fed cholesterol, and it has been shown that the plaques appear to retrogress when cholesterol is withdrawn from the diet Various attempts have been made to inhibit the deposition of cholesterol in the aorta of cholesterol fed chicks and rabbits and Turner (12) showed that the incorporation of thyroid into the diet of cholesterol fed rabbits resulted in a certain degree of prophylaxis against atherosclerosis Before attempting to produce inhibition or retrogression of the lesions in the rabbits of our laboratory

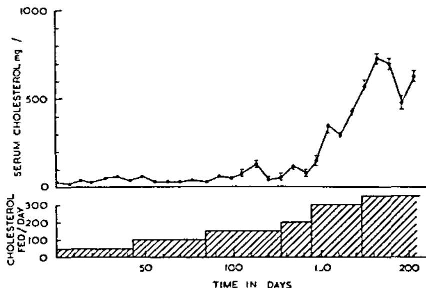


FIG 5 The effect of cholesterol intake on the serum cholesterol in the rabbit.

strain, we decided to investigate the susceptibility of our colony to cholesterol feeding In the first experiment, 6 male and 6 female rabbits were put on a diet which contained fixed amounts of cholesterol in olive oil in the standard rabbit diet. Over a period of about 6 months the cholesterol content of the diet was gradually increased, and it was found that below the level of 200 mg/rabbit/day there was little influence on the serum cholesterol of the animals Above this value the serum cholesterol of the rabbits rose quite sharply and as a result of this experiment it was decided to adopt a feeding regimen of 300 mg cholesterol in olive oil per day It can be seen from the results of this experiment (Fig 5) that the serum cholesterol of rabbits on this diet "plateaus" at about 600 mg/100 ml. of serum In this experiment it was found that the female rabbits were more suspectible to cholesterol feed

ing than the males insofar as the females on the same diet usually had serum cholesterol levels about 30% greater than those prevailing in the males In order to test whether triac administration could result in protection of rabbits from cholesterol induced atherosclerosis the following experiment was set up

Female Chinchilla rabbits were fed 300 mg cholesterol in olive oil per day in the standard rabbit diet These animals were bled at frequent intervals and after 120 days on this diet were separated into two

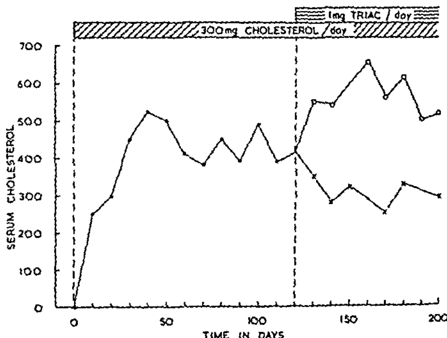


Fig 6 The effect of triac on the serum cholesterol of cholesterol fed rabbits

groups each of 12 animals The grouping was arranged so that animals with comparable serum cholesterol levels were placed in opposite groups The control group continued to consume the diet containing cholesterol while the test group consumed the cholesterol diet together with 1 mg triac/animal/day The test animals lost weight throughout the experiment and the serum cholesterol levels of the control and the test groups were measured as before It was found that the introduction of triac to the diet of these cholesterol fed rabbits resulted in a rapid drop in the serum cholesterol level and this drop was fairly well maintained throughout the experiment as is shown in Fig 6

It was found necessary to adjust the dose of triac administered to certain animals in the test group As a rule this was accomplished by

giving the animal half the usual dose of triac for about a week before returning the animal to the same dose as the rest of the group

On four occasions during the triac administration period certain animals exhibited severe toxic side effects which disappeared when the drug was withheld for about 3 days One animal died during the test period

After 120 days on this diet the animals were killed and the cholesterol contents of the serum liver and aorta measured in the usual way In addition samples of various organs and the aortic arch and the heart were sent for histological examination and the findings will be reported elsewhere It was found that the liver cholesterol of the triac treated animals was reduced to 30% of that prevailing in the control group and the aortic cholesterol of the triac treated animals was only 60% of that prevailing in the control group We conclude therefore that triac can partially inhibit the deposition of cholesterol in the aorta of cholesterol fed rabbits of this particular strain This result is not contrary to the published observation of Pitt Rivers and Trotter (9) who attempted to assess the efficiency of triac on the regression of atheroma in rabbits previously fed cholesterol In their study triac was shown to be inactive in increasing the rate of retrogression of atheroma in control against triac treated animals It would seem therefore that in the cholesterol fed rabbit this thyroxine analog is able to prevent arterial lipid deposition presumably by modifying the circulating cholesterol level The problem of whether this substance is active in removing existing atherosclerotic lesions remains in doubt

SUMMARY

1 In this strain of rats the induction of hypothyroidism by I^{131} ablation of the gland or by surgical excision depresses the rate of hepatic cholesterol synthesis without producing a marked plasma hypercholesterolemia

2 By contrast chemical induction of hypothyroidism by 0 3% dietary thiouracil produces a depression of hepatic cholesterol synthesis and an elevated plasma cholesterol level which suggests an extrathyroidal effect of thiouracil

3 The thiouracil effect can be obtained in the absence of the thyroid and appears to be intensified in the presence of a dietary cholesterol supplement suggesting that the drug interferes with cholesterol degradation and excretion

4 Studies on thyroxine triiodothyronine tetrac and triac on cholesterol metabolism and tissue oxygen consumption in the rat have been presented The thyroactive substances studied elevated the oxygen

consumption rate of the rats elevated the rate of hepatic cholesterol synthesis but had little effect on the plasma cholesterol level in this species In these hyperthyroid animals the ventricular oxygen consumption rate rose more rapidly than any other tissue examined

5 The administration of one thyroactive compound (triac) to cholesterol fed rabbits resulted in depression of the plasma cholesterol level and partial inhibition of aortic cholesterol deposition

ACKNOWLEDGMENT

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REFERENCES

1 Barker S B *Endocrinology* 59 548 (1956)
2 Boyd G S and Oliver M F *Bull Swiss Acad Med Sci* 13 384 (1957)
3 Bruce H M *J Hyg* 48 171 (1950)
4 Ciba Colloquium Vol 10 (1957)
5 Cuthbertson W F J Elcoate P V Ireland D M Mills D C B and Shearley P *Proc Biochem Soc* 25P (1958)
6 D Amour F E and Blood F R Manual for Laboratory Work in Mammalian Physiology Univ Chicago Press Chicago Illinois 1948
7 Gildea E F Man E B and Peters J P *J Clin Invest* 18 739 (1939)
8 Maclagan N F and Sheahan M M *J Endocrinol* 6 456 (1950)
9 Pitt Rivers R and Trotter W R *Brit J Exptl Pathol* 38 97 (1957)
10 Sperry W M and Webb M *J Biol Chem* 187 97 (1950)
11 Tomich E G and Woollett E A *J Endocrinol* 11 134 (1954)
12 Turner K B *J Exptl Med* 58 115 (1933)

DISCUSSION

MARMORSTON *I would like to corroborate the work of Dr Boyd This work will be reported tomorrow We also used thiouracil alone and thiouracil and cholesterol as a standard method for producing hypercholesteremia and atherosclerosis in dogs and the administration of these two materials raises the serum cholesterol to high levels just as it does in the rat The administration of cholesterol in thyroidectomized dogs raises the serum cholesterol to still higher levels and also produces widespread atherosclerotic lesions I would like to ask Dr Boyd whether he has tried the effect of estrogens in rats on serum cholesterol hypercholesteremia or atherotic lesions in the vessel*

BOYD We have very little experience of rendering rats atherosclerotic but we have looked at the effects of estrogens on the serum cholesterol of normocholesteremic rats Among adult male rats receiving 50 μg hexestrol per 100 g body weight per day within 5 days the serum cholesterol level is depressed to 50% of the initial value We have used this type of experiment as an assay procedure in which the animals received the drug subcutaneously per day and after 8 days the serum cholesterol is determined and then the drop in serum cholesterol is expressed as a percentage With stilbestrol or hexestrol within the dose range 5 to 50 μg per 100 g body weight, the serum cholesterol response is related to the log of the dose

STRISOWER Studies done on euthyroid individuals with l thyroxine and tri iodothyronine at the Donner Laboratory have shown a strong correlation between initial serum lipoprotein and serum cholesterol concentration and the decrease in these levels brought about by thyroid hormones In other words the higher the initial serum lipoprotein concentration particularly of the S_f 0–20 class the greater is the decrease in these levels produced by a given dose of l thyroxine and l triiodothyronine I wonder if you have made an analysis by separating the individual rats with respect to their pretreatment cholesterol levels and have been able to show whether or not those rats that have high initial levels may show some even though slight effect compared to those that have low initial levels and if not I might suggest that perhaps the lack of effect might be due to the very low initial serum cholesterol levels found in the rat I should like to ask two questions I notice that in your experiments with rabbits you have studied female rabbits only and I wonder if the effect of triac is the same in male rabbits? We did find an interesting sex difference in humans when studying l triiodothyronine which I shall show later in detail I just wonder whether such a sex difference exists in rabbits Looking at your serum cholesterol curves in rabbits I noticed that the cholesterol level increased for a period of 50 days which seems a rather long period of time and that in the control group following the administration of triac there was a further increase Would you care to comment on the significance of these findings?

BOYD At maturity the serum cholesterol levels of our rats show little deviation from the control level so that we rarely see rats which spontaneously show serum cholesterol levels say double the control value Thus I cannot really tell you whether drugs are more effective in animals spontaneously showing an elevated serum cholesterol level The second point, I used females in this series because the female is more susceptible in this Chinchilla strain to hypercholesterolemia than is the male We start with 24 female rabbits fed cholesterol and pair off the animals so that the total serum cholesterol of the groups are identical

STRISOWER What was the size of individual groups in terms of numbers of animals?

BOYD Twelve in each

STRISOWER Has it been your experience that feeding cholesterol will cause an increase in cholesterol levels for a period of 50 days and then some maybe for 100 days such as the curve shows? Is that found in other strains? It seems a very long period especially for a small animal

BOYD We have used this Chinchilla strain as a test animal and I have not had a great deal of experience with other strains We merely set out to establish if a drug which could depress the serum cholesterol level would also decrease the amount of cholesterol deposited in the vessels

RALL I think it is very interesting that Dr Boyd has shown this extrathyroidal effect of thiouracil which was perhaps first hinted some years ago when Barker found that the thiouracil treated rat required a much larger amount of thyroxine to bring the oxygen consumption back to normal than did the thyroidectomized rat I think this peripheral effect may well explain some of the studies that were not clear a few years ago on the effect of thyroidectomy versus thiouracil on enzyme concentrations in liver and other tissues It is often difficult to interpret in the whole animal changes in serum cholesterol because the timing and the dosage and the dose response curve of the particular thyroxine analog being studied are of great importance So frequently we have seen in the human that with almost any thyroxine analog one chooses one can get a relatively rapid fall in serum cholesterol in normal

myxedematous or familial hypercholesterolemic persons but nearly always there is in spite of continued administration of the thyroxinelike compound a gradual increase in the serum cholesterol Depending upon when one analyzes the group one can get all sorts of different findings

BERGSTROM Your data have brought to mind some results that we have obtained in collaboration with Dr Bengt Gustafsson on his germ free rats In one experiment the normal controls had a serum cholesterol of 77 mg % whereas the germ free animals on the same diet had an average of 110 mg % i e as large a difference as between your normals and your thiouracil treated rats This is another example of the pronounced influence of the intestinal flora What happens to the serum cholesterol in man during prolonged treatment with antibiotics? It might perhaps be profitable to modify the intestinal flora under certain conditions

DRILL With regard to Dr Marmorston s question on estrogens Dr Cook in our laboratories is also studying the effect of estrogens on serum cholesterol in the rat and obtains effects quite comparable to those of Dr Boyd Would you discuss further the toxic effects obtained in the rabbit?

BOYD The main toxic effect is that the animals stop eating and they exhibit a tremor nervousness restlessness and this disappears when the drug is withheld These triac treated animals had a body weight of about 2 5 kilos and they lost 500 to 700 g during the experiment

ADLERSBERG In connection with the effect of bacteria on cholesterol metabolism one should perhaps consider the bulk content of the diet as an important nutritional factor affecting lipid metabolism There are some old observations that a high bulk diet is a decholesterolizing agent whereas a bland low bulk diet has the opposite effect One could even think that some of the important differences in the diet between the Bantus and the Europeans are based on different fat and protein intake and perhaps different intake of dietary bulk as well Now I wonder whether increased shedding of cells and loss of cholesterol in the colon an area where the cholesterol cannot be reabsorbed couldn t be the effective factor of a high bulk diet It is otherwise difficult to understand the effect of the high bulk diet I would like to ask Dr Boyd whether he studied any other lipids than cholesterol in his rats In rabbits fed 1 g of cholesterol per day we have seen a leveling off of serum cholesterol and phospholipid levels and of total lipids after approximately 2 to 3 months

BERGSTROM Certainly the bulk of the diet has a very pronounced influence also in humans If you use the emulsions of Ahrens you have a very small amount of feces If you add bulk in rats there is also a very marked effect These germ free rats and their controls of course had sterilized food

ADLERSBERG Was there any difference in the diets of the sterile rats and those infected? I am referring to carbohydrates proteins fats minerals and bulk

BERGSTROM The whole diet being used is sterilized inside the germ free cage and then the food for the control animals is transferred to the outside

BOYD As a rule the only observation we make with regard to the serum is the cholesterol level and occasionally phospholipids The electrophoretic lipoprotein method which I use on human plasma is not very suitable for use with rat plasma because the precision with which we can measure the α β ratio falls off at very low plasma cholesterol levels

CHAPTER 5

The Conversion of C^{14} Acetate to Cholesterol in the Euthyroid and Hypothyroid Dog

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In 1915 Kendall (3) described a crystalline compound containing iodine which occurs in the thyroid its chemical nature and physiological activity The next year in summarizing extensive studies of cholesterol in human physiologic and pathologic states Luden (5) observed that three patients with myxedema showed extremely high serum cholesterol levels Furthermore this hypercholesterolemia could be reversed by the administration of either Kendall s "alpha iodine compound" or thyroxine Such were the early bases for the hypothesis that serum cholesterol levels are regulated by the secretion of a specific endocrinologic chemical compound

The fact that the "regulatory" property of the thyroid is undoubtedly indirect and involves a complicated metabolic process is rather immaterial to those of us who are utilizing thyroid inhibition as a tool in the study of atherosclerosis It remains a fact that the hypothyroid dog exhibits much of the pathology and biochemistry of the human atherosclerotic In a series of articles beginning in 1946 Steiner Kendall and Bevans (1 7 8) demonstrated that the cholesterol fed thiouracil treated dog was atherosclerotic in a manner distributionally and microscopically similar to the human disease that increased serum cholesterol concentrations paralleled and were well correlated with the development of arterial and cerebral lesions that the feeding of thiouracil alone will induce early lesions in puppies and that serum lipoprotein changes in such an animal are consistent with similar observations in the human

Our own interest concerns that portion of the atherosclerotic process which involves deposition of cholesterol at the wall of the artery We have expended considerable laboratory time in an effort to devise a system which would definitively demonstrate the relative tendencies of the various serum lipoprotein classes to either (*1*) deposit *in toto* at the arterial wall or (*2*) transfer their cholesterol to such contiguous tissue (With respect to this discussion the mechanisms are interchangeable) There is substantial indirect evidence to indicate that provided that cholesterol deposition from the blood has any bearing at all on the

pathogenesis of atherosclerosis such a difference in tendency does in fact exist In reporting his studies of three South African population segments Keys (4) noted that the electrophoretically determined serum β lipoprotein concentration was well correlated with the incidence of coronary disease Gofman (2) has made extensive simultaneous measurements of high (alpha) and low (beta) density lipoproteins in humans who had suffered myocardial infarction and has been unable to identify any relationship between the serum concentration of the high density classes and disease—in sharp contrast to his findings with the β or low density lipoprotein classes Our own experience with a more limited series of coronary diseased humans has been identical (6) It would seem profitable therefore provided that the proper experimental situation can be established to demonstrate such differences of serum lipoprotein behavior *in vivo* and to evaluate them quantitatively

Obviously the greatest obstacle to establishing a quantitative relationship between the change of a blood lipoprotein concentration and any corresponding cholesterol deposition at the arterial wall is the practical impossibility of satisfactory artery biopsy However along with other investigators in this field we had considered the possibility of utilizing C^{14} acetate as a means of establishing and measuring cholesterol accumulation in tissue It would seem *a priori* that if an animal were given the isotope labeled acetate *de novo* the specific activity of cholesterol isolated after 24 hours from the coronary artery for instance would be in itself a rate measurement of cholesterol deposition in that tissue Such is the case of course but only with significant reservations Primarily the specific activity of cholesterol isolated from any tissue is a function of the size of the acetate pool from which the cholesterol is synthesized If that pool is small and a given quantity of C^{14} acetate is administered to the animal the specific activity of that acetate pool will be relatively high and subsequently regardless of the number of intermediate steps (mevolonic acid squalene etc) the cholesterol synthesized from that pool will have a relatively high specific activity The converse is true of course if the acetate pool is large

Furthermore there is no reason to believe that there is any constancy of pool size among animals nor that any treatment (such as I^{131} administration) will or will not alter the pool size to any degree subject to calibration Measurement of the pool size (by simultaneous administration of sulfanilamide for instance) is impractical because it would be necessary to prove that sulfanilamide is acetylated and cholesterol is synthesized from the same pool Even if the "pool" obstacle could be overcome or avoided the problem of dilution would exist Assuming increased deposition in the hypercholesterolemic state there

would be the same increase of inactive cholesterol and active cholesterol deposited thus little if any change in specific activity could be observed Furthermore assuming the possibility of calibrating and validating a change of small magnitude the important matter of timing would require thorough prior investigation It is obvious that the specific activity of cholesterol isolated from the artery could be a measure of deposition only during that period wherein such specific activity is increasing or precisely at its peak *in situ* At any subsequent time the specific activity of the isolated cholesterol would become an important function of the cholesterol catabolic activity in the animal and the investigator would be faced with another pool problem in reverse Finally the important technical problems described by Schwenk (9) and others involving high counting companions of cholesterol isolated from tissue by digitonide precipitation would require extensive preliminary study One could not expect to recover enough material from a 300 mg (wet weight) coronary artery for purification through the dibromide and other requisite chemical manipulations With these difficulties in mind the following assumptions were made

1 Mechanism of acetate C^{14} incorporation → cholesterol is independent of thyroid activity thus

$$\text{Observed cholesterol specific activity} = \frac{\text{Number } C^{14} \text{ atoms}}{\text{Total } (C^{1} + C^{14}) \text{ atoms}} = D \frac{\text{Number tagged cholesterol molecules}}{\text{Total cholesterol molecules}}$$

2 The largest portion of arterial cholesterol results from active or passive transfer from contiguous blood

3 The mechanism of blood tissue transfer is independent of thyroid activity

4 1 g artery tissue = 1 cc

Apparently each item is either precisely true within the errors of the measurements that are involved or is a statement that fails to contradict current theory or any existing data In addition our hypothesis requires a fundamental basis in fact of two experimental observations

1 All cholesterol *in vivo* exists and is transported as lipoprotein

2 "High counting companions of cholesterol precipitable by digitonin are absent 24 hours after acetate 2 C^{14} administration to dogs

Both are reasonable and are accepted as fact by most investigators Thereafter the general equation for the physiochemical model that relates the specific activities of cholesterol isolated from serum and arterial tissue could be derived (Eq 1)

C = serum cholesterol concentration mg/g
W = artery cholesterol concentration mg/g
S = observed specific activity serum cholesterol counts/min/mg
T = observed specific activity artery cholesterol counts/min/mg
D = conversion factor atoms to molecules
a = proportion of low density lipoprotein cholesterol
k_H and k_L = affinity constants for high and low density lipoprotein cholesterols

In the serum

(*a*) Total lipoprotein cholesterol concentration

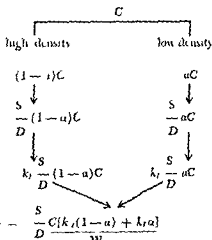
(*b*) Lipoprotein fraction cholesterol concentration

(*c*) Tagged cholesterol concentration by density fractions

In the artery wall

(*d*) Tagged cholesterol concentration by density fractions

(*e*) Cholesterol specific activity



rearranging
$$\frac{T}{S} = \frac{C}{W}\left[k_H + (k_L - k_H)a\right] \qquad (1)$$

This rather simple arithmetic relationship contains five quantities that could be readily measured (S T C W and a) and two unknowns (k_H and k_L) It was hoped that by utilizing two experimental animals the measured quantities could be respectively substituted and these equations solved simultaneously It was decided to use a modified dog preparation of the Steiner Kendall Bevans type as the experimental animal Cholesterol feeding was eliminated because of the fatty liver which results Since the liver is a prime site of cholesterol synthesis such obvious liver pathology might negate assumptions 1 and 3 above In our hands thiouracil administration to adult dogs took a considerable period to induce high serum lipoprotein concentrations and therefore I^{131} was substituted in most recent studies Preliminary experiments had indicated that the administration of I^{131} resulted in a quantitatively variable serum lipoprotein response in dogs but in each instance there

was a prompt and sustained hyperlipoproteinemia involving all lipo protein classes

Certain auxiliary studies were undertaken in order to evaluate the validity of the derivation expressed in Eq (1) The data in Figs 1 and

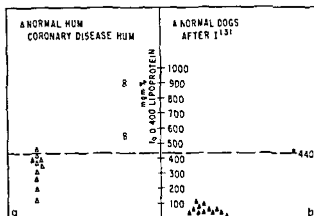


Fig 1 Serum lipoprotein concentration in (*a*) disease free and atherosclerotic humans and (*b*) dogs before and after I^{131} administration

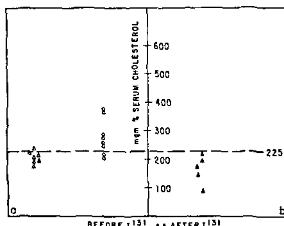


Fig 2 Serum cholesterol levels in (*a*) the same human subjects as in Fig 1*a* and (*b*) the same dogs as in Fig 1*b*

2 indicate that serum lipoprotein and cholesterol concentration in dogs before and after I^{131} administration are altered in a manner similar to the differences observed between presumably disease free and athero sclerotic humans The common ordinate in these scattergrams is the sum of standard S_f 0–400 serum lipoproteins as determined ultra

centrifugally The open circles in Fig 1*a* represent values obtained on 10 coronary disease patients the open triangles 10 randomly selected West Point cadets (not only free from manifest disease but also screened for healthy cardiovascular systems from the general population) from a group of some 600 The closed triangles and circles in Fig 1*b* are corresponding lipoprotein measurements on a group of 10 dogs before and 24 weeks after the administration of I^{131} The horizontal line is drawn at the 440 mg % level

The point is strikingly obvious These data are not meant to hold any special brief for screening power of lipoprotein measurements nor

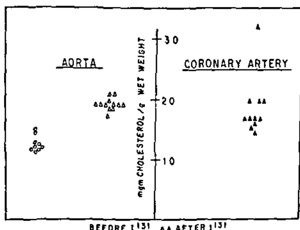


Fig 3 Scattergrams of dog aortae and coronary arteries before and after I^{131} administration

their relationship to coronary disease but merely one of the many relationships that support study of atherosclerosis in the hypothyroid dog Simple serum cholesterol determinations on the same individuals and dogs as seen in Figs 2*a* and 2*b* indicate similar aspect with the horizontal delineation at 235 mg %—perhaps not quite as obvious The scattergram recorded as Fig 3 demonstrates that after I^{131} administration *the wall of dog aortae and coronary arteries will in fact accumulate* cholesterol The decay curves for specific activities of high and low density lipoprotein cholesterols are shown in Fig 4 It is noted that as required in the derivation of Eq (1) the specific activities of the two lipoprotein classes come to equilibrium after 12 hours

The data contained in Table I reveal some interesting facts Standard doses of acetate 2 C^{14} were administered to 2 dogs Plasma cholesterol levels and the specific activities of the total cholesterol the β lipoprotein cholesterol and the α lipoprotein cholesterol were re

corded at their maximum points 1 hour after administration of the labeled acetate Note that the specific activities of the isolated cholesterol fractions are not greatly in variance in spite of the fact that the cholesterol concentration in Dog #1 was roughly 60% of that recorded for Dog #2 Subsequently I^{131} was administered to both animals and after 30 days cholesterol concentration and specific activity values

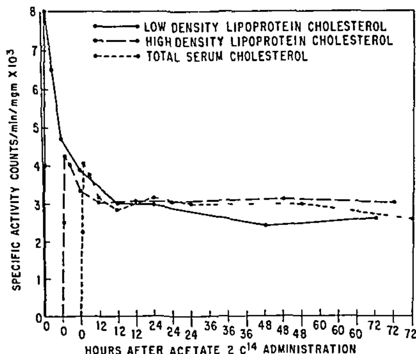


FIG 4 Decay curves for specific activities of high and low density lipoprotein cholesterols

were again recorded It has been our preliminary observation that the lipoprotein response to I^{131} is somewhat more pronounced in animals that initially have higher serum cholesterol levels

These data are examples of such response in that the serum cholesterol concentration of Dog #1 showed virtually no significant increase whereas after I^{131} administration the serum cholesterol concentration in Dog #2 more than doubled The dilution effect of this increased serum cholesterol concentration on the specific activity of the total serum cholesterol as well as the high and low density lipoprotein cholesterol fractions is strikingly obvious As a matter of fact, in Dog #2 with roughly a 200% increase in cholesterol concentration the

specific activity of the isolated serum cholesterol decreased from 4200 counts/min/mg to 1480 counts/min/mg In that animal therefore there was not only a dilution effect but also an obvious expansion in acetate pool size In Dog #1 on the other hand, specific activity data revealed a contraction in acetate pool size as a result of I^{131} administration as indicated by the significant increase of cholesterol specific activity *It should be noted that after 30 days cholesterol isolated from the* serum of each dog still revealed significant activity

TABLE I
CHOLESTEROL CONCENTRATION AND SPECIFIC ACTIVITY IN 2 DOGS 1 HOUR AFTER STANDARD DOSES OF ACETATE 2 C^{14} ("before") AND 30 DAYS AFTER I^{131} ADMINISTRATION (after)

	Dog #1		Dog #2	
	Before	After	Before	After
Plasma cholesterol	1 13	1 21	1 79	3 71
Maximum specific activity				
serum 1 hour	*4790*	*6120*	*4200*	*1480*
β Fraction 1 hour	*10 700*	*11 200*	*8350*	*1450*
α Fraction 1 hour	*4920*	*6200*	*4380*	*1520*
Background	*0*	*332*	*0*	*586*

Table II details our early attempts at collecting the data necessary to solve for the affinity constants set forth in Eq (1) Of the two dogs *involved #1 was sacrificed after 10 weeks of thiouracil feeding* The data were collected on Dog #2 30 days after the oral administration of 10 mc of I^{131} in a single dose All we can say about these results and *similar values obtained the same way is that they are all positive num* bers between 0 and 1 and they reveal *k* values for the low density *lipo protein cholesterol that are higher than the corresponding k values for* high density cholesterol

There is no theoretical reason why affinity constants for coronary artery aorta and femoral artery *for high and low density lipoprotein* cholesterol should be identical As a matter *of fact we have repeatedly* observed a higher coronary artery cholesterol concentration than aortal *cholesterol concentration in the same dog Such would indicate different* affinity constants for the two tissues *On the other hand based on our* theoretical model the cholesterol derived from a single lipoprotein class *should have the same affinity constant for corresponding tissues between* dogs Such has not been observed thus far We have speculated that our failure in this regard is due to the fact that we have divided all the lipoproteins into two large classes and assumed identical distribution of subclasses within the high and low density fractions Such assumption is obviously not warranted *However we feel that the approach is*

TABLE II
DATA AND RESULTS—HIGH AND LOW DENSITY LIPOPROTEIN AFFINITY CONSTANTS
(k_H AND k_L OF EQ 1)

Dog	Serum cholesterol		Coronary artery cholesterol		Aorta cholesterol		Femoral artery cholesterol		Proportion low density lipoprotein cholesterol
	C	S	W	T	W	T	W	T	α
	Conc	S A [b]	Conc	S A	Conc	S A	Conc	S A	
#1 Thiouracil	1 42	1640	1 40	600	1 34	319	0 95	342	0 135
#2 I[131]	3 71	1260	1 67	1090	1 35	1150	0 98	767	0 468
Solution for									
k_H			0 35		0 12		0 13		
k_L			0 44		0 57		0 20		

[a] Milligrams per gram wet weight
[b] Counts/min /mg

sound and that by further refinement of our analytical technique and measurements accomplished on narrower density fraction of serum lipoproteins we will be able to establish some valid estimate of the relative tendencies of the varying lipoprotein moieties to deposit at the wall of the artery

References

1 Bevans M Davidson J D and Kendall F E *Am Heart J* 38 462 (1949)
2 Gofman J W personal communication (1955)
3 Kendall E C *J Am Med Assoc* 64 2042 (1915)
4 Bronte Stewart B Keys A and Brock J F *Lancet* 269 1103 (1955)
5 Luden G *Collected Papers Mayo Clin and Mayo Foundation* 10 429 (1918)
6 Milch L J unpublished data (1955-1958)
7 Steiner A and Kendall, F E *A M A Arch Pathol* 42 433 (1946)
8 Steiner A Kendall F E and Bevans M *Am Heart J* 38 34 (1949)
9 Schwenk E and Werthessen N T *Arch Biochem Biophys* 42 91 (1953)

Discussion

STRISOWER I wonder if you could define for me the terms k_H and k_f?

MILCH Let us consider an area of artery intimal surface equal to 1 gram of tissue If then of every ten high density lipoprotein molecules that pass that area three either adhere to the intimal surface or pass their cholesterol content to the intimal surface k_H would equal 0 3

HOLMAN First I would like to congratulate Dr Milch on his efforts to get some contact with the blood vessel wall I am still old fashioned enough to believe that atherosclerosis has something to do with the blood vessel wall and I want to compliment him on making this deliberate attempt to do so The manner in which lipids accumulate in the blood vessel wall is of paramount importance to all of us We should be able to devise methods properly to assess the relative importance of filtration local formation or other mechanisms by which lipids accumulate in the arterial wall Dr Milch discussed this The question which I would like to ask Dr Milch is has he found any differences in different segments of different coronary arteries or what segment does he routinely use for this purpose?

MILCH For chemical analyses we are unable to talk about segments—there is little enough of the dog coronary artery already Actually the coronary is divided near the ostium and that portion circling the top of the dog heart is analyzed for cholesterol concentration and cholesterol specific activity The portion that descends and branches along the wall of the heart is reserved for histological examination

HOLMAN The one at the top which runs around the heart, is the right coronary artery You apparently sent the anterior descending branch of the left coronary artery to the pathology laboratory Did they find any anatomic changes or did they make radioautographs that might give any lead as to the site of deposition?

MILCH In coronaries taken from animals maintained for 1 year after I^{131} administration there seemed to be some increased oil red O deposition

FREEDBERG The right coronary artery in the dog is very small Dr Holman so that he was probably getting the left circumflex

HOLMAN Possibly

KATZ Dr Milch you have made at least four assumptions as the basis of your mathematics Have you any independent evidence that the assumptions are true

because if any one of them turns out to be false your calculations would not be valid

MILCH You will recall that I stated that each item was rather precisely true within the errors of measurements that are involved or was a statement that failed to contradict current theory or existing data Suppose we consider the assumptions in order

1 That "the mechanism of acetate C^{14} incorporation into cholesterol is independent of thyroid activity such that the equation

$$\text{Cholesterol specific activity} = \frac{\text{Number of } C^{14} \text{ atoms}}{\text{Total } (C^{1} + C^{14}) \text{ atoms}} = D \frac{\text{Number of tagged cholesterol molecules}}{\text{Total cholesterol molecules}}$$

remains true" Obviously the equation is precise without regard to thyroid activity The only question is whether or not the term "D is altered as a function of thyroid activity I know of no data to indicate such alteration will occur

2 That "the largest portion of arterial cholesterol results from active or passive transfer from contiguous blood Again I know of no data to contradict such an assumption In support however we have the experiments carried out by Dr Gould some years ago which indicate that *in vitro* at least the uptake of C^{14} acetate and synthesis of cholesterol by arterial tissue is very slow indeed

3 That the mechanism of blood tissue transfer is independent of thyroid activity" Here again I know of no data to contradict such an assumption I will concede of course that the rate of such transfer may very well be thyroid dependent

4 That "1 gram of artery tissue = 1 cc No comment necessary

KATZ Another assumption is that blood tissue transfer is independent of the thyroid

MILCH As I pointed out it is difficult to see why the mechanism should change The *rate* of transfer of course is undoubtedly a complicated function of the many metabolic alterations that are associated with change of thyroid function

KATZ The last assumption is that cholesterol is in lipoprotein and is distributed among the family of lipoproteins in the same manner in the presence as in the absence of thyroid in hypothyroid euthyroid and hyperthyroid states

MILCH Such was not an assumption We demonstrated that the distribution of lipoproteins among the density classes is altered in a qualitatively predictable manner as a result of I^{131} administration

KATZ But the cholesterol in the several types of lipoproteins is not redistributed Does the thyroid have any effect on the cholesterol content of each type of lipoprotein?

MILCH I hadn t considered that point However I don t believe that a redistribution of the cholesterol portion of the lipoprotein molecule is necessary for the validity of our theoretical model

WERTHESSEN I can t quarrel with Dr Milch s assumptions even though he feels that the transfer of cholesterol in the hypercholesterolemic animal may be passive I think it doesn t really make very much difference for the study whether it is an active or a passive transfer The thing that has fascinated me in your data is the apparent repetition of a finding in the perfused aorta You apparently approach a

sound and that by further refinement of our analytical technique and measurements accomplished on narrower density fraction of serum lipoproteins we will be able to establish some valid estimate of the relative tendencies of the varying lipoprotein moieties to deposit at the wall of the artery

REFERENCES

1 Bevans M Davidson J D and Kendall F E *Am Heart J* 38 462 (1949)
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CHAPTER 6

The Relationship between Thyroid Hormones and Cholesterol Biosynthesis and Turnover[1]

R Gordon Gould

Los Alamos Scientific Laboratory University of California Los Alamos New Mexico

At the present time perhaps the most promising approach to the prevention of atherosclerosis would be to lower the plasma cholesterol level in preatherosclerotic humans for prolonged periods of time The effects of diet, hormones and other measures on the plasma cholesterol level are most frequently studied in postcoronary patients where there can be little hope of accomplishing more than the slowing down or arresting of the atherogenic process simply by decreasing the plasma cholesterol level after advanced and presumably irreversible lesions are already present Such drastic measures as estrogen therapy or subsistence on homogenized diets of purified components require great interest and cooperation on the part of the patient and it is too much to expect that large groups of young normal men would cheerfully submit to them for years to see if any of these measures will prevent the development of atherosclerosis and its sequelae Consequently it is of interest to investigate the mechanisms regulating the plasma cholesterol level in normal as well as in hypercholesterolemic states with the hope of finding a practicable method of lowering the plasma level in "normal" preatherosclerotic people for indefinite periods

There is a vast literature on the effects of everything imaginable on the plasma cholesterol level but very little in the way of an explanation of how the effects are brought about In recent years there has been a good deal of work done on methods of inhibiting cholesterol synthesis in liver with the hope of thereby lowering the plasma level This hope is based on the implicit assumption that the plasma level is regulated by the rate of synthesis in liver an assumption that is hardly warranted by the evidence While it is undoubtedly true that a complete inhibition of cholesterol synthesis without inhibition of its catabolism must eventually result in a drop in plasma level, it does not follow that a controlled decrease in rate will result in a corresponding decrease in level In fact a decreased rate of cholesterol biosynthesis is frequently associated with an increased plasma level as appears to be the case in hypothyroidism

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[1] The portion of the work carried out at Los Alamos was under the auspices of the United States Atomic Energy Commission

maximal level of cholesterol in the thyroidectomized dogs You show a large range of concentration in your control animals In the experimental animals you show a clumping on the high side of the normal values Now may not that indicate that you have to put in another assumption here or add another factor that would express a tendency to limit the amount of cholesterol that could accumulate in the tissue If some such factor is not operating you should have seen the same range in your treated animals as you did in your controls rather than a grouping at a high level of the control values I think that if you developed your equation a little bit further and brought this factor into consideration you might get rid of some of the variation you are seeing in your results

MILCH I agree Aortal and coronary artery cholesterol concentrations listed in Fig 3 were recorded at 1 year after I^{131} administration and were undoubtedly at the maximum level of accumulation in the arteries of the I^{131} treated dogs In other words a new equilibrium had been reached under the hormonal and blood lipoprotein levels that had been attained However for purposes of determining the affinity constants k_H and k_I we measure artery cholesterol concentration and specific activity 30 days after I^{131} administration where the actual additional mass accumulation of cholesterol is almost nil and could not in fact have reached the equilibrium levels dictated by the hormonal and blood lipoprotein levels extant at that time

WERTHESSEN The beginning of the inhibition of accumulation is a function of the original level You don t know the original level unfortunately because you have not done biopsies

MILCH Such is true of course for any individual dog On the basis of control and I^{131} dog groups however no significant difference between arterial cholesterol concentration can be demonstrated

viously reported (8) specific activity values for the plasma free cholesterol 4 hours after administration of 100 μc of acetate 1 C^{14} were 0 024 μc per g of cholesterol for the spontaneous myxedema group 0 016 for the secondary group and 0 128 for the control euthyroid group The plasma levels were approximately twice as high in the myxedema patients as in controls

Table I shows the effect of replacement therapy on the incorporation of acetate C^{14} into plasma free cholesterol of 6 myxedema patients Each patient was given two 100 μc doses of acetate C^{14} one before

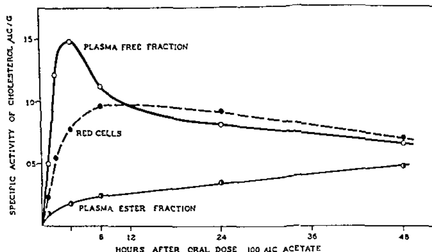


FIG 1 Rates of equilibration of cholesterol C^{14} derived from hepatic biosynthesis in blood fractions The mean values for 7 normal human subjects are shown

treatment and a second one after a period of several weeks or months of treatment The specific activity values 4 hours after acetate administration were on the average about 5 times as high when the patients were euthyroid as when they were myxedemic

It is customary to correct the specific activity values for changes in the "pool" size and to express the synthetic rate in terms of free cholesterol C^{14} contained in a given volume of plasma We do not consider this a valid procedure because the whole pool of cholesterol in equilibrium with plasma free cholesterol must be taken into account as discussed below However since the specific activity values were 5 to 6 times as high in the euthyroid state and the plasma levels about half as high correction for the increased plasma level in this manner would suggest that the rate of synthesis in myxedema is decreased to about one half to one third of the normal rate Lipsky *et al* (13) found

The hypercholesterolemia resulting from hypothyroidism or myxedema is at present without explanation but it is, at any rate not due to an increased rate of biosynthesis of cholesterol in liver In fact as Karp and Stetten first showed (11) there is a decreased rate of incorporation of deuterium from body water into cholesterol in hypothyroid and an increased rate in hyperthyroid rats This dependence on thyroid hormone activity was found to be not specific for cholesterol biosynthesis in liver but applied to biosynthetic processes generally in all body tissues The magnitude of the effect on liver cholesterol biosynthesis was about 20% for both hypo and hyperthyroid states Most subsequent investigations have confirmed both of these effects in experimental animals (2 3 14) and in man (8 13) although the amount of change observed has varied considerably and some workers have not found significant decreases in hypothyroid animals (5) or human subjects (10)

Dr George LeRoy and his associates at the University of Chicago and our group at the Los Alamos Scientific Laboratory have been carrying on collaborative studies on various aspects of cholesterol metabolism in humans by means of radioactive tracers since 1952 I would like to present some of our results on the effect of myxedema on cholesterol synthesis in liver

The methods used were based on previous animal studies in which we found that the rate of synthesis in liver could under certain specific conditions be estimated by determining the specific activity of plasma free cholesterol The rate of mixing or equilibration of free (i e unesterified) cholesterol C^{14} between liver and plasma in a dog following intravenous injection of acetate 1 C^{14} is so rapid that at any time after the first hour the specific activity of plasma free cholesterol is approximately equal to that of liver free cholesterol and is a reliable measure of it (4) We have a few data from human liver biopsies that show the same type of relationship and indicate that free cholesterol in liver and plasma reach equilibrium in less than 4 hours The rates of appearance of newly synthesized cholesterol in plasma free erythrocyte free and plasma esterified cholesterol fractions in 9 normal humans following a standard 100 μc oral dose are shown in Fig 1 Intravenous injection gave essentially identical results It is evident why it is important to measure the specific activity either of plasma free or whole blood free cholesterol rather than total—esterification is a slow process

When untreated myxedema patients are compared by this technique with euthyroid subjects or with the same patient following replacement therapy the effect of myxedema on the rate of cholesterol biosynthesis in liver may be estimated In a series of 10 myxedema patients pre

the total plasma volume by 23% and the plasma volume per kilogram of body weight by 28.5% Blumgart *et al* (1) found a decreased plasma volume in myxedema and Gibson and Harris (6) found a plasma volume

TABLE II
ESTIMATION OF THE FREE CHOLESTEROL CONTENT OF THE LIVER BLOOD POOL IN EUTHYROID AND MYXEDEMIC PATIENTS

Type patient	Cholesterol concentration Free (mg/ml)	Cholesterol concentration Total (mg/ml)	Cholesterol content in 70-kg man Free (g)	Cholesterol content in 70-kg man Total (g)
Euthyroid				
Plasma	0.61	2.02	1.7	5.6
Erythrocytes	1.50	1.50	3.4	3.4
Liver	2.55	3.00	4.6	5.4
			9.7	14.4
Myxedema				
Plasma	1.42	4.74	3.1	10.4
Erythrocytes	1.50	1.50	2.7	2.7
Liver	2.93	4.20	5.3	7.6
			11.1	20.7

Increase in myxedema = 14% This estimation is based on the following assumptions

(1) Blood volume is 7.1% of body weight when euthyroid and 20% lower than this weight when hypothyroid (see text)

(2) Hematocrit is 0.45 and not appreciably changed in myxedema For a 70-kg standard man this results in

	Euthyroid	Myxedema
Plasma volume (ml)	2750	2200
Erythrocyte mass (g)	2250	1800

(3) Plasma cholesterol is 30% free in both euthyroid and in myxedematous states

(4) Liver weight is 2.6% of body weight or 1800 g for a 70-kg subject when euthyroid and does not change in myxedema The cholesterol concentration is 3.0 mg per g of which 85% is free in the euthyroid state

(5) Liver free cholesterol concentration is assumed to increase by 15% in myxedema and total by 40%

in myxedema patients 15% smaller than predicted For the purpose of this estimate we have taken a 20% reduction as probable

The red cell volume has also been reported to be lower in myxedema (17) and by about the same amount as plasma volume We have assumed a reduction of 20% in erythrocyte cholesterol content It may

a 5 fold decrease in untreated myxedema after correcting for changes in the plasma cholesterol pool size in this way

The pool of cholesterol in equilibrium with plasma free cholesterol 4 hours after acetate C^{14} administration consists primarily of 3 components liver free erythrocyte free and plasma free cholesterol frac

TABLE I

EFFECT OF REPLACEMENT THERAPY ON INCORPORATION OF ACETATE C^{14} INTO PLASMA FREE CHOLESTEROL IN MYXEDEMA PATIENTS[a]

Case number	Clinical status	Plasma free cholesterol specific activity (μc/g)	Plasma total cholesterol level (mg/ml)
1	Before therapy	0 030	6 80
	D thyroxine for 22 days	0 127	2 85
2	Before therapy	0 038	4 00
	L thyroxine for 20 days	0 161	1 30
3	Before therapy	0 037	3 35
	Desiccated thyroid for 6 months	0 187	2 25
5[b]	Before therapy	0 020	4 60
	L thyroxine for 80 days	0 132	2 45
7[b]	Before therapy	0 015	6 20
	L thyroxine for 57 days	0 055	1 66
9	Before therapy	0 035	3 50
	L thyroxine for 54 days	0 160	1 60
	Mean before therapy	0 029	4 74
	Mean after therapy	0 137	2 02
	Ratio after/before	4 7	0 43

[a] One hundred μc of acetate C^{14} was injected intravenously and blood samples were taken 4 and 24 hours later Values above are for 4 hour samples the 24 hour samples had lower specific activity values in all cases but the changes due to replacement therapy were similar

[b] Acetate 2 C^{14} was used in both studies on these patients and acetate 1 C^{14} in all others

tions The effect of myxedema on the amounts of cholesterol in these three compartments is difficult to estimate satisfactorily primarily because of the lack of knowledge of the amount of free cholesterol in liver Table II gives estimates for a standard 70-kg human based on the average values for the plasma cholesterol levels of the group of 6 myxedema patients before and after treatment and on a number of assumptions

Although the plasma cholesterol level increases in myxedema the plasma volume decreases appreciably Thompson (17) in a thorough study of 9 myxedema patients found that replacement therapy increased

The change in cholesterol synthetic rate during replacement therapy in myxedema patients was associated with a concomitant increase in BMR (basal metabolic rate) and a decrease in plasma cholesterol level Figure 2 illustrates an attempt to see if either of these variables could be shown to be responsible for the change in synthetic rate No striking relationship between synthetic rate and either BMR or plasma cholesterol level is apparent in this case Studies in rats and rabbits have led us

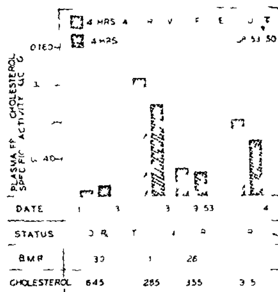


Fig 2 Effect of intermittent replacement therapy on acetate C^{14} incorporation into plasma free cholesterol on BMR and on plasma total cholesterol level in a patient with myxedema

The first pair of values was obtained before any therapy was given the second after D-thyroxine had been administered daily for 3 weeks the third after a 4 week period of no therapy and the fourth after L thyroxine administration for 3 weeks The changes in incorporation do not appear to follow closely the changes in either BMR or plasma cholesterol level

to the conclusion that a decrease in the rate of synthesis in liver is almost always associated with a slight increase in liver cholesterol concentration but unfortunately we were not able to obtain liver biopsies from these patients and have no information on possible cholesterol concentration changes

Rates of disappearance of labeled cholesterol from plasma have been determined on a number of these patients and on some normal humans by injecting or feeding cholesterol H^3 Examples are shown in Fig 3 In a euthyroid human injected intravenously with 33 8 μc of cholesterol

be presumed for lack of evidence to the contrary, that the absolute liver weight is not affected by myxedema The marked decrease in body weight after replacement therapy is instituted is largely due to excretion of water rather than to tissue loss The effect of myxedema on the liver cholesterol concentration would be of considerable interest to know but it does not seem to be established whether or not there is any change in humans In experimental animals reported data suggest no significant changes at least not in rats For example Karp and Stetten (11) found no changes in the liver cholesterol level in rats about 3 weeks after being given thiouracil May *et al* (15) found no changes after thiourea (or thyroxine) administration for 14 to 60 days and Frantz *et al* (5) no change after I^{131} administration Handler (9) reported an increase in rat liver cholesterol resulting from thyroidectomy but since the animals were maintained on a high cholesterol diet the conclusion does not apply to normal diets However, Milch *et al* (16) have recently reported an increase of about 40% in total liver cholesterol in dogs kept for a year or longer after treatment with I^{131} Whether this discrepancy is due to species differences or to the longer period of observation is not certain Only total cholesterol concentrations were determined so the increase in free cholesterol is not known Ordinarily increases in liver cholesterol are very largely due to increases in the esterified fraction as this fraction like the esterified fraction in plasma does not come to equilibrium to an appreciable extent with plasma or liver free cholesterol in 4 hours it is not a part of the cholesterol pool under consideration In cholesterol fed rats and rabbits the liver esterified cholesterol level rises but the specific activity resulting from acetate C^{14} administration falls to negligible values in this fraction

In Table II the increase in the free cholesterol pool is estimated on the assumption that a 40% increase occurs in total cholesterol in liver from 3 0 to 4 2 mg per g but that the increase in free cholesterol is only about 15% from 2 55 to 2 93 The rest of the excess cholesterol is assumed to be present in esterified form Although this assumption cannot be justified by human data *it is consistent with the distribution of* cholesterol in liver of various species of animals under conditions which result in elevated liver cholesterol levels

If the above estimate is correct the correction for change in "pool" size to be applied to the specific activity value amounts to only 14% Consequently we consider the specific activity value of plasma free cholesterol to be a better indicator of the change in the rate of hepatic cholesterol synthesis in myxedema than the corrected" value obtained by multiplying the specific activity by the *plasma* concentration of free (or total) cholesterol

H^3OH The criticism is often made that the incorporation of acetate C^{14} added as a very small amount of acetate with high specific activity does not measure the rate of synthesis but only incorporation To control the possibility that a diversion of acetate to or from other channels of metabolism or that changes in the endogenous acetate "pool" may be

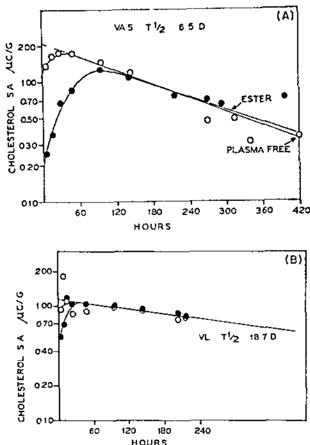


FIG 3 Rates of disappearance of intravenously injected cholesterol H^3 from plasma free and plasma esterified cholesterol fractions in a euthyroid human (A) and in a myxedema patient (B)

responsible for changes in incorporation we have studied the effect of varying the amount of acetate C^{14} added to liver slices In the range from 15 μmoles of acetate C^{14} per g of liver slices to 150 μmoles the specific activity of the cholesterol isolated varied only from 4 0 $\times$ 10^4 to 4 9 $\times$ 10^4 DPM/mg (disintegrations per minute per milligram) with the maximum value at 70 μmoles and minimum values at both ends

H^3 the plasma cholesterol specific activity decreased for about 2 weeks with a half time of about 6 5 days In a myxedema patient injected with 39 2 μc of cholesterol H^3 the half time was 18 7 days during the period of observation (which was only 8 days) or almost 3 times as long This effect of hypothyroidism which is in agreement with reports of others (12 18) emphasizes the often neglected fact that catabolic and excretory processes are slowed down in hypothyroidism as much if not more than biosynthetic ones

It is becoming apparent to many investigators that it is incorrect to speak of *a* turnover time or "*a* pool size" for cholesterol The specific activity of plasma cholesterol changes with time due to equilibration with cholesterol present in most body tissues as well as by excretion and catabolism of cholesterol in the liver There are many "pools" of cholesterol which interchange with plasma cholesterol at varying rates and to varying degrees of completeness Consequently the observed turnover half time as shown in Fig 3 is an arbitrary concept representing the sum total of a number of turnover and mixing processes It applies only during the particular period shown and its principal usefulness is for detecting marked changes in various disease states

The size of the cholesterol "pool is equally arbitrary Injected labeled cholesterol will decrease in specific activity due to dilution with free cholesterol present in plasma liver erythrocytes spleen and all visceral organs at a fairly rapid rate and with the large pools in skin muscle and blood vessel walls more slowly The size of the "pool at 7 days has been estimated to be 26 to 55 g It is considerably smaller at 4 and 24 hours after injection but the size has not been estimated in humans at these time intervals after the injection of normal plasma lipoproteins containing labeled cholesterol In dogs the application of this technique indicates a "pool of about 3 to 4 times as much cholesterol as is present in blood at 24 hours after injection (7)

So many studies have been reported on factors affecting the rate of cholesterol synthesis as determined by the incorporation of acetate C^{14} into liver slice cholesterol that it is of interest to see if the implicit assumption that rates *in vitro* are an accurate index of rates in the intact animal under all circumstances is really valid

One exception is fasting which appears to decrease the rate of synthesis in liver slice incubations with or without added glucose more than in intact rats We have found a decrease to less than 20% of the control value in liver slices from rats fasted for 48 hours and to about 50% in intact rats For this reason we believe it important to use both methods and to compare under the same conditions the incorporation of acetate C^{14} with that of other labeled precursors for example

H^3OH The criticism is often made that the incorporation of acetate C^{14} added as a very small amount of acetate with high specific activity does not measure the rate of synthesis but only incorporation To control the possibility that a diversion of acetate to or from other channels of metabolism or that changes in the endogenous acetate "pool may be

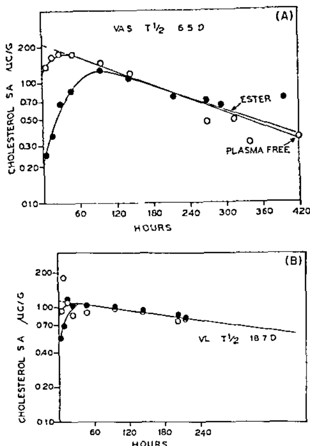


FIG 3 Rates of disappearance of intravenously injected cholesterol H^3 from plasma free and plasma esterified cholesterol fractions in a euthyroid human (A) and in a myxedema patient (B)

responsible for changes in incorporation we have studied the effect of varying the amount of acetate C^{14} added to liver slices In the range from 15 µmoles of acetate C^{14} per g of liver slices to 150 µmoles the specific activity of the cholesterol isolated varied only from 4.0×10^4 to 4.9×10^4 DPM/mg (disintegrations per minute per milligram) with the maximum value at 70 µmoles and minimum values at both ends

of the range Definitely lower values were found in the range from 3 5 to 9 μmoles The relative constancy above 15 μmoles per g supports the assumption that in this range we are dealing with an essentially infinite reservoir of labeled precursor and that the actual rate of synthesis is being measured

In the intact animal an infinite reservoir of acetate can obviously not be used without pharmacological effects However we have studied the effect of fasting and X irradiation on cholesterol synthesis using both acetate 1 C^{14} and H^3OH in the same animals and for the same length of time The cholesterol isolated from liver gave specific activity ratios of C^{14} to H^3 varying from 0 505 to 0 548 in four groups of 3 rats each with C^{14} specific activity values varying from 785 DPM/mg to 6020 The fasted control group showed slightly lower ratios with a mean of 0 37

Some years ago Frantz and his associates (5) suggested that the inhibitory effect of ingested cholesterol on cholesterol biosynthesis in liver could be expressed as an inverse relationship between total cholesterol concentration in liver and the log of the synthetic rate in liver slices As dietary cholesterol accumulates in liver its distribution between free and esterified forms changes remarkably with almost all the increase occurring in the esterified fraction It seems more probable that either the free or the ester form rather than the sum of the two is inhibitory

If all the data we have collected in *in vivo* studies are put on one plot one obtains reasonably good agreement with a linear relationship for free cholesterol concentration but not for total (Fig 4) or for esterified cholesterol The most rapid rates of synthesis have been noted in livers from irradiated rats in which a decreased level of liver cholesterol was invariably found This effect has been established by both *in vivo* and *in vitro* methods, using both acetate C^{14} and H^3OH it is also apparent in homogenates Only total cholesterol analyses have been done routinely in these experiments but the ratio of free to esterified is relatively constant in the range below about 3 mg per g of liver thus in this range a linear relationship is found for both free and total cholesterol Above this value the synthetic rate is decreased In cholesterol fed rats free and total cholesterol were studied separately and a *linear relationship between free cholesterol concentration and the* log of the synthetic rate was found but the total followed a curve approximating that shown for adrenalectomized and for hypophysectomized animals We have not yet studied the effect of these two procedures on the distribution of cholesterol between free and esterified

forms in liver but the total concentration was found to increase considerably as the rate of synthesis decreased to very low levels

A little progress has been made toward locating the rate determining step in cholesterol biosynthesis In collaboration with Dr George Popjak a comparison of the rate of cholesterol biosynthesis as measured by acetate C^{14} and by mevalonic acid 2 C^{14} (3 5 dihydroxy 3 methyl valeric

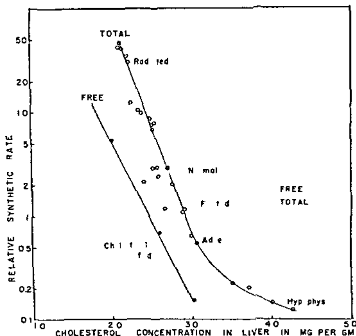


Fig 4 Relationship between logarithm of the relative synthetic rate of cholesterol synthesis in rat liver *in vivo* and the concentrations of free and total cholesterol in liver

Each point is the mean for a group of 3 to 6 animals Experiments were carried out on rats treated as follows control fasted irradiated fasted (300–2400 r) cholesterol fed adrenalectomized adrenalectomized fasted adrenalectomized irradiated fasted hypophysectomized hypophysectomized fasted hypophysectomized irradiated fasted

acid 2 C^{14}) was made under various conditions X irradiation does not increase the rate of synthesis of cholesterol in liver homogenates from mevalonic acid 2 C^{14} nearly as much as from acetate 1 C^{14} even when a large excess of substrate is present as shown in Table III

It is of interest that homogenates prepared from livers of fasted control rats gave no evidence of cholesterol biosynthesis from either substrate Thus irradiation does counteract the inhibitory effect of fasting

but does not give the extremely high rates of synthesis from mevalonic acid (MVA) as from acetate

TABLE III
CHOLESTEROL BIOSYNTHESIS FROM ACETATE 1 C^{14} AND FROM MEVALONIC ACID 2 C^{14} AS INFLUENCED BY X IRRADIATION

Substrate	Amount of substrate (μmoles per g of liver)	Rat liver homogenates[a] Fed controls (DPM/mg)	Irradiated and fasted for 48 hours (DPM/mg)
Acetate 1 C^{14}	31	200	3440
	93	190	2530
Mevalonic acid 2 C^{14}	1	3350	5070
	3	5830	8850
	10	4940	4390

[a] Each value represents the mean of 6 rat livers Homogenates prepared essentially as described by Bucher and incubated 2 hours

Cholesterol feeding decreases synthesis from acetate many times more than from mevalonic acid (Table IV) In this *in vivo* experiment the results expressed as C^{14} in the cholesterol present in a gram of liver tissue (to correct for the large increase in concentration in the cholesterol fed rats) show a decrease to less than 3% of the control rate as measured by acetate incorporation and a decrease to 25% of the control rate as measured by mevalonic acid incorporation

TABLE IV
CHOLESTEROL BIOSYNTHESIS FROM ACETATE 1 C^{14} AND FROM MEVALONIC ACID-2 C^{14} AS INFLUENCED BY CHOLESTEROL FEEDING IN INTACT RATS

Tissue	Relative synthetic rate mμc of C^{14} in cholesterol per g tissue Control[a]	Cholesterol fed[b]	Ratio of control to cholesterol fed
Liver Acetate	1 60	0 044	36
MVA	2 96	0 72	4
Intestine Acetate	1 03	1 43	0 7
MVA	0 12	0 02	5 7
Carcass Acetate	0 083	0 040	2 1
MVA	0 026	0 014	1 8

[a] Control diet commercial rat chow + 10% corn oil
[b] Cholesterol diet control diet + 1% cholesterol
Diets were fed for 7 days and rats were injected I P with 10 μc of acetate 1 C^{14} or 0 18 μc of mevalonic acid 2 C^{14} per 100 g of body weight and sacrificed 4 hours later Each value represents 6 rats

Table IV also shows the meager incorporation of MVA into cholesterol by intestine and also by the rest of the carcass It is apparent from these figures that the apparent relative rates of cholesterol synthesis in various tissues will depend on what substrate is used This effect may be due to a lower permeability of extrahepatic tissues than liver to MVA However the possibility is still open that there may be significant differences in cholesterol biosynthesis in various tissues It is evident that the rate of cholesterol biosynthesis in liver varies over a thousandfold range (as the result of cholesterol feeding or whole body X irradiation) with little if any change in rate in intestine and many other tissues

In conclusion I would like to venture the suggestion that the rate of cholesterol metabolism in liver may be controlled in part by two feedback mechanisms as indicated in Fig 5 When the concentration

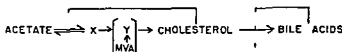


Fig 5 A suggested mechanism of control of cholesterol metabolism in liver
One feed back mechanism highly sensitive to the concentration of unesterified cholesterol in liver appears to control the rate of cholesterol biosynthesis primarily by acting on some reaction between acetoacetyl CoA and mevalonic acid (MVA) or possibly an unidentified compound closely related to MVA
A second feed back mechanism sensitive to bile acid concentration in liver may control the rate of conversion of cholesterol to bile acids

of cholesterol in liver rises slightly the rate of synthesis falls markedly and conversely Further Bergstrom and his associates have recently suggested that the main catabolic pathway of cholesterol in the rat the conversion to bile acids may be controlled by a similar feedback mechanism Removal of bile acids as in bile fistula animals greatly accelerates the conversion of cholesterol to bile acids and bile acid administration inhibits this conversion

A rate controlling step in cholesterol biosynthesis appears to be between acetoacetyl CoA and either MVA itself or an activated form of MVA The rate determining step in bile acid formation as Dr Bergstrom has suggested is probably the first hydroxylation resulting in the introduction of the 7α hydroxyl group

References

1 Blumgart H L Cargill S L and Gilligan D R *J Clin Invest* 9 91 (1930)
2 Byers S O Rosenman R H Friedman M and Biggs M W *J Exptl Med* 96 513 (1952)

3 Dayton S Dayton J Drummer F and Kendall F E *Circulation* **10** 595 (1954)
4 Eckles N E Taylor C B Campbell D J and Gould R G *J Lab Clin Med* **46** 359 (1955)
5 Frantz I D Jr Schneider H S and Hinkelman B T *J Biol Chem* **206** 465 (1954)
6 Gibson J C II and Harris A W *J Clin Invest* **18** 59 (1939)
7 Gould R G in Proc Ann Meeting Council High Blood Pressure Research p 3 American Heart Assoc International Press New York 1952
8 Gould R G LeRoy G V Okita G T Kabara J Keegan P and Bergenstal D M *J Lab Clin Med* **46** 374 (1955)
9 Handler P *J Biol Chem* **173** 295 (1948)
10 Hellman L and Rosenfeld R S personal communication (1958)
11 Karp A and Stetten D Jr *J Biol Chem* **179** 819 (1949)
12 Kurland G S and Lucas J L *J Clin Invest* **34** 947 (1955)
13 Lipsky S R Bondy P K Man E B and McGuire J S Jr *J Clin Invest* **34** 950 (1955)
14 Marx W Gustin S T and Levi C *Proc Soc Exptl Biol Med* **83** 143 (1953)
15 May L G Moseley R W and Forbes J C *Endocrinology* **38** 147 (1946)
16 Milch L J Renzi A A Weina N Robinson L G and Wilson S S *Proc Soc Exptl Biol Med* **97** 56 (1958)
17 Thompson W O *J Clin Invest* **2** 477 (1926)
18 Weiss S B and Marx W *J Biol Chem* **213** 349 (1955)

Methyltestosterone and the Thyroid

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We shall not present any data on cholesterol synthesis or rates of cholesterol synthesis in various situations having arrived here more or less through the backdoor because of our interest in the thyroid and in particular in the thyroid hormone protein complex that exists in plasma and the rather interesting relationship between sex steroids and the specific protein in plasma which binds thyroxine and triiodothyronine I would like to review briefly how we can estimate the level of this specific protein (TBP thyroxine binding protein) and what happens to the level of this protein during treatment with estrogens and methyltestosterone and how the total thyroid activity changes

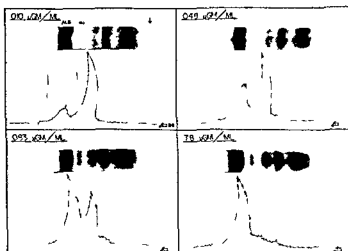


FIG 1 Paper electrophoretic patterns of normal human serum to which varying quantities of I^{131} labeled L thyroxine have been added The stained paper strip is at top and the record of radioactivity below it in each case (From *J Clin Invest* **34** 1325 1955)

Figure 1 shows the distribution of varying amounts of labeled thyroxine added to human serum and subjected to paper electrophoresis The paper electrophoretic patterns are shown above the radioactivity which is thyroxine I^{131} is shown below and you can see that at low and

physiologic concentrations of thyroxine about 80% of it is located just between alpha 1 and alpha 2 globulin and perhaps 20% travels with albumin At increasing concentrations of thyroxine more and more is found to be associated with albumin

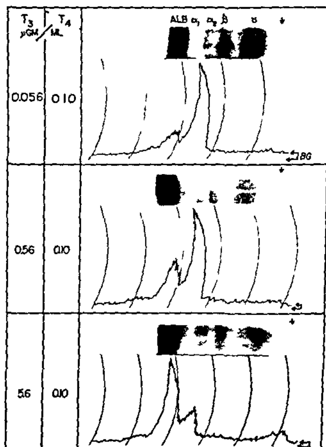


FIG 2 The effect of addition of varying quantities of triiodothyronine on the distribution of labeled thyroxine in normal serum (L triiodothyronine + L thyroxine I^{131} in normal serum) (From *J Clin Invest* **34** 1333 1955)

Formerly the limiting amount of thyroxine which could be associated with TBP in any given serum was impossible to quantitate Recently however one of us by use of a simple modification of paper electrophoresis *has been able to arrive fairly accurately at the saturation value* of TBP for thyroxine Figure 2 demonstrates that triiodothyronine will also combine with TBP As shown in Fig 2 the thyroxine was labeled and varying quantities of unlabeled triiodothyronine were added It

can be seen that with increasing quantities of triiodothyronine there is displacement of thyroxine from TBP to albumin Similar data have been presented by Albright *et al* using labeled triiodothyronine itself We have estimated as a first approximation that triiodothyronine is bound about one third as firmly to TBP as is thyroxine

Table I reviews briefly some physical data on TBP Its electrophoretic mobility in barbital buffer at pH 8 6 places it just between alpha 1 and alpha 2 globulin The mobility is very similar in phosphate buffer at pH 7 In acetate chloride buffer pH 4 5 TBP is still negatively charged and migrates with the M 2 group of alpha globulins Characteristically some of these glycoproteins are soluble in perchloric acid but TBP or at least the thyroxine associated with it is precipitated

TABLE I

PROPERTIES OF THE THYROXINE BINDING ALPHA GLOBULIN OF SERUM (TBP)

Electrophoresis pH 8 6	$\mu \cong -4\,7 \times 10$ cm volt ^{1} sec ^{1}
Electrophoresis pH 4 5	$\mu \cong -1\,7 \times 10^{5}$ cm volt ^{1} sec ^{1}
Sedimentation	$S_{\,)w} \cong 3\,3$
Solubility in potassium phosphate buffer pH 6 5	88% ± 4 6 at 1 40 M buffer
	62% ± 6 4 at 2.10 M buffer
	8 2% ± 2 8 at 2 80 M buffer
Plasma protein fractions (Cohn Method No 6)	Fraction IV-4
	? Fraction VI 3

by this reagent The sedimentation of thyroxine in the ultracentrifuge gives a value of 3 3 S_{0w} a value somewhat lower than that of albumin Unfortunately diffusion data are not available for the accurate determination of its molecular weight but if TBP has a shape similar to that of albumin its molecular weight would be about 50 000 The solubility in phosphate buffer which is not particularly characteristic is included because thyroglobulin which has a very similar electrophoretic mobility shows a very sharp range of insolubility in this buffer When serum is fractionated by the Cohn method most of TBP seems to be in fraction IV-4 A few data which are not shown but which can be calculated are as follows The level of total thyroxine in normal human serum is approximately 10^{7} *M* the level of sites for thyroxine on TBP is about $2\,6 \times 10^{7}$ *M* the association constant of thyroxine for TBP we have estimated to be about 8×10^{9} This last value depends on figures in the literature for the association constant of thyroxine and bovine serum albumin and is subject to considerable uncertainty The level of free thyroxine in serum subject to similar uncertainties can be cal

culated to be $6 \times 10^{11} M$ assuming equilibrium conditions It may be however that metabolism and/or excretion of serum free thyroxine is more rapid than its dissociation from the protein so that the calculated equilibrium value does not reflect actual serum levels In this instance the rate limiting step could be dissociation of thyroxine from TBP

I shall not present any of the data we have adduced on the relation ship between the concentration of free thyroxine in blood and the metabolic effect and degradation of the thyroid hormone Suffice it to

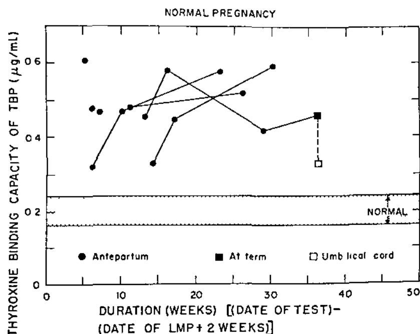


FIG 3 Level of thyroxine binding protein in serum during pregnancy (From *Recent Progress in Hormone Research* **13** 194 1957)

say at this point that data on subjects with hyperthyroidism hypothyroidism and nephrosis are compatible with the notion that it is the level of free thyroxine as distinct from the bound thyroxine which is important Figure 3 shows that pregnancy elevates the level of TBP and this occurs very early in pregnancy and reverts to normal 5 or 6 weeks after the termination of pregnancy There is a concomitant increase in the PBI (protein bound iodine) of serum As a result of these changes the level of free thyroxine in blood remains within the normal range These conclusions have also been reached by Ingbar *et al* and have been extended by them to show that estrogen administration reproduces this effect Indeed when estrogens are administered to an athyreotic subject

receiving desiccated thyroid the expected rise in TBP occurs but there appears to be no change in PBI and the subjects show evidence of hypothyroidism We might explain this on the basis of a decreased level of free thyroxine Unfortunately there are no good data available to indicate the precise activity of the thyroid gland and rate of metabolism of thyroxine in these conditions

We became interested in the effect of testosterone on thyroidal activity when Keitel and Sherer reported last year that methyltestosterone administered to dwarfs lowered the serum PBI It occurred to us

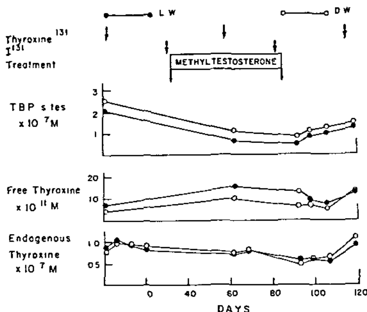


FIG 4 Effect of methyltestosterone on certain parameters of thyroxine metabolism

that this might be mediated through effects on thyroxine binding protein Table II shows the data on 4 essentially normal individuals who received 100 mg of methyltestosterone daily for 7 weeks It can be seen that as far as the thyroid itself is concerned there were inconsistent changes in iodine clearance There were similarly no consistent changes in the renal clearance of iodide Table III shows the effect of methyl testosterone on the serum protein bound iodine There is a consistent albeit minor fall in all four cases Accompanying this are striking decreases in the level of TBP in serum There is consequently in general an increase in the calculated level of free thyroxine in serum Figure 4 shows the results of serial determination of these parameters in two of the individuals and gives some idea of the consistency that one may

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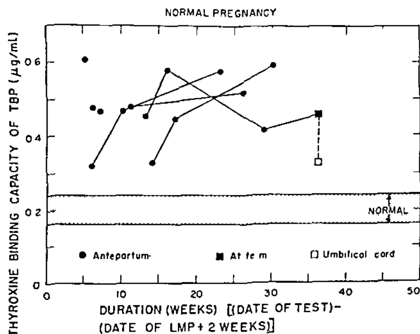


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TABLE III

THE INFLUENCE OF METHYLTESTOSTERONE ON IODINE METABOLISM PROTEIN BOUND IODINE THYROXINE BINDING PROTEIN AND FREE THYROXINE LEVELS IN SERUM

	B B		C H		L W		D W	
	Control	Methyl testosterone (29)	Control	Methyl testosterone (28)	Control	Methyl testosterone (29)	Control	Methyl testosterone (29)[a]
Endogenous thyroxine[b] $\times 10^{-7}$ M	1.4	0.90	0.77	0.44	0.89	0.71	0.78	0.71
TBP sites for thyroxine $\times 10^{-7}$ M	3.0	1.5	1.7	0.66	2.1	0.67	2.6	1.1
Free thyroxine $\times 10^{-11}$ M	8.9	10	7.3	8.2	7.1	16	4.6	9.9

[a] Number of days on testosterone at time of study
[b] Calculated from the PBI on the assumption that it consists entirely of thyroxine
[c] Conversion factor PBI (μg %) = 5.03 × 10^7 × molar concentration thyroxine

TABLE II
INFLUENCE OF METHYLTESTOSTERONE ON THYROIDAL UPTAKE AND THYROIDAL AND RENAL CLEARANCE OF IODIDE

	B B		C H		L W		D W	
	Control	Methyl testos terone	Control	Methyl testos terone	Control	Methyl testos terone	Control	Methyl testos terone
Radioiodide uptake %								
3 Hours	29	16	20	9 8	12	12	15	16
6 Hours	36	21	18	12	14	14	17	22
24 Hours	—	—	24	13	19	18	27	33
Thyroidal iodide clearance[a]								
ml /min /1 73M	39	20[a]	20	20	9 4	13	8 0	18
Renal iodide clearance[a]								
ml /min /1 73M	43	35	56	100	43	36	27	34

The clearances were calculated using a correction for the extrathyroidal neck background obtained by giving a second tracer to each patient after blocking with sodium iodide Such a tracer could not be given to BB his clearance was therefore calculated by applying to the 2 minute neck reading the average correction shown necessary by the other patients studies

which rose slightly during treatment with testosterone This was correlated reasonably well with a slight increase in body weight which occurred simultaneously

We have then the following data to correlate A fall in the serum level of thyroxine a normal or increased rate of degradation of thyroxine and a normal thyroid clearance of iodide The most logical hypothesis to explain these findings appears to us to be that the primary effect of methyltestosterone is to cause a reduction in the level of TBP

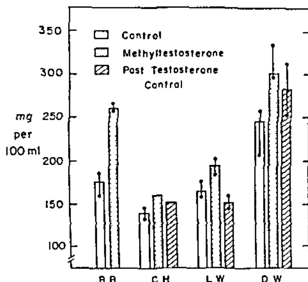


FIG 5 Effect of methyltestosterone on serum cholesterol (From *J Clin Invest* 37 1024 1958)

in serum Initially this would be expected to cause an increase in the level of free thyroxine in serum This could be expected to increase the rate of degradation of thyroxine which would eventually cause a fall in the serum PBI The elevated level of free thyroxine might also cause a decreased secretion of TSH (thyroid stimulating hormone) which would temporarily inhibit thyroidal activity Eventually one would expect a new steady state to be reached at which time there would be a lower than normal TBP a depressed serum thyroxine normal thyroid activity an essentially normal rate of degradation of organic iodine and a normal level of free thyroxine Since we were unable to examine all these parameters at very frequent intervals it is impossible to be sure that this was the exact sequence of events but it appears to us at this writing to represent the most reasonable theory In all events these

expect from data of this sort It can also be seen that even 40 days after cessation of methyltestosterone administration the level of TBI has not returned to normal Table IV shows the effect of methyl testosterone on thyroxine metabolism Thyroxine labeled with I^{131} was given intravenously and its rate of disappearance from the plasma measured for 2 to 3 weeks It can be seen that in all four subjects there

TABLE IV
THE INFLUENCE OF METHYLTESTOSTERONE ON IODINE METABOLISM RADIOTHYROXINE DISAPPEARANCE RATE ORGANIC IODINE POOL AND ORGANIC IODINE DEGRADATION RATE

Patient	Status	PBI[b]	Thyroxine disappear ance rate[c]	Extra thyroidal organic iodine pool[d]	Organic iodine degradation[e]
BB	Control	7 2	11	590	65
	Methyltestos terone[26] [f]	4 6	16	450	70
CH	Control	3 8	14	390	55
	Methyltestos terone[27][f]	2 2	22	390	85
	Control[159][f]	4 0	13	490	64
LW	Control	4 5	15	340	50
	Methyltestos terone[23][f]	3 6	18	300	54
	Control[30][f]	4 6	12	380	45
DW	Control	4 0	13	330	43
	Methyltestos terone[23][f]	3 6	19	330	61
	Control[30][f]	5 6	9 9	500	50

From *J Clin Invest* 37 1024 1958
[b] Micrograms 100 ml serum
[c] Per cent per day
[d] Micrograms iodine/1 73M
Micrograms iodine degraded/day/1 73M
[f] Number of days on testosterone or off testosterone at time of study

was an unequivocal increase in the fractional rate of loss of thyroxine from the plasma during administration of methyltestosterone In the three subjects in which thyroxine disappearance was measured after the cessation of treatment with testosterone the fractional rate returned toward normal Since there was an accompanying fall in the PBI only relatively minor changes in the rate of organic iodine degradation cal culated on the basis of micrograms per day per unit surface area oc curred Not listed in Table IV is the volume of distribution of thyroxine

venous infusion of C^{14} labeled cholesterol (4 C^{14}) in a patient who unfortunately died 15 days after we gave him the material intravenously The specific activity in the skin was 50% of that of the plasma and liver which were in equilibrium at that time The aorta incidentally also had a lower specific activity than the plasma

DRILL Dr Rall mentioned that the calculated amount of free thyroxine increased after methyltestosterone administration Did the amount of free thyroxine increase or decrease after the administration of estrogen during pregnancy?

RALL There is a slight decrease in pregnancy There is a great deal of overlap in the values but the mean free thyroxin in a group of 20 or 30 pregnant women is significantly below the mean normal free thyroxin level This occurs because the TBP rises slightly more than the serum thyroxin

DRILL Would you have expected the reverse effect in that the calculated free thyroxine might have gone up during estrogen administration and decreased during methyltestosterone administration? If this occurred one might be able to correlate the effects of estrogen cholesterol and methyltestosterone

RALL Yes To correlate cholesterol effects one would guess just the reverse would happen but it seems to me quite likely that estrogen and methyltestosterone affect cholesterol directly and independently of the thyroid

KATZ Dr Rall have you any data on the effect of sex hormones on hyper thyroid or hypothyroid patients?

RALL There are some data that I think Kinsell had some years ago on the effect of treatment of hyperthyroid individuals I think he had two cases treated with testosterone propionate There was a mild increase in nitrogen retention and perhaps mild amelioration of symptoms It is difficult to say In one or two cases in which he treated hyperthyroid individuals with methyltestosterone he noted some exacerbation of the disease This would make good sense to us if there were a decrease in TBP in this situation

ADLERSBERG I would like to ask two questions I am interested in a concept that factors of distribution of cholesterol and other lipids between blood and tissues the liver especially and perhaps the skin are more important than we usually think in studying circulating lipid levels Occasionally one doesn't know where this cholesterol comes from There is for example the observation by Friedman and Byers in San Francisco that after injection of large doses of triglycerides in rats a rapid elevation of serum cholesterol occurs It cannot be synthesized so rapidly It represents probably a shift from some tissues to the blood Interestingly enough this happens also in hepatectomized animals Apparently the source of this rapid shift of cholesterol must be in some other tissues perhaps the skin I do not know how important the depot of skin cholesterol is The second brief question is Did I understand you correctly Dr Gould that the equilibration between plasma and liver occurs more rapidly in the rat than in the dog? Is this correct and have you any studies in other species e g the rabbit? How would you explain these species differences?

GOULD I make no claim to be able to explain species differences but there certainly are some that are quite striking The rabbit is slower than the dog and generally more variable than other animal I have studied The specific activity of plasma free cholesterol takes about 3 hours to reach a peak value in humans and only about 1 hour in dogs but the shape of the curve is identical The few values for the specific activity of free liver cholesterol that we have obtained for humans at very short time intervals after the administration of acetate confirm the general idea that the liver free cholesterol reaches its peak specific activity first and the plasma free

data show that sex steroids can influence thyroid hormone activity by extra thyroidal means

Figure 5 shows the effect of methyltestosterone on the serum cholesterol It can be seen that in all subjects this drug caused a significant increase in the level of serum cholesterol which returned towards normal when the drug was discontinued Oliver and Boyd have noted *similar results in hypercholesteremic patients treated with methyl testosterone* It is of interest that at first glance the effects of sex hormones on cholesterol might appear to be mediated via the thyroid (assuming that increased thyroid activity is indicated by a rise in PBI and will cause a depression of serum cholesterol) Estrogens elevate the PBI of serum and lower the cholesterol—methyltestosterone depresses the PBI and raises serum cholesterol The data presented however seem to indicate that other changes in the thyroxine binding protein of serum are such as to maintain a relatively normal level of free thyroxine in blood which may be the critical factor in thyroidal effects Therefore the effects of sex steroids on serum cholesterol are most probably direct effects

DISCUSSION OF PAPERS BY DRS GOULD AND RALL

MILCH Relative to Dr Rall s findings we have some indication that the blood lipid and lipoprotein response to methyltestosterone is variable among humans In collaboration with Colonel Groover our laboratory has made extensive blood lipid measurements within a large group of human males over a 3-year period In many instances we recorded marked diminutions of blood cholesterol concentration after methyl testosterone administration

RALL I would have been much happier to see a fall in the serum cholesterol since this would fit our theories better—all I can say is we didn t In view of the thyroid studies I think we must conclude that the effect of methyltestosterone on cholesterol was direct

WERTHESSEN I think that in this human work you ought to take into consideration that the human is a peculiar animal in that it has an enormous amount of fat under the skin There is a possibility therefore that if you corrected for the amount of subcutaneous fat change in your patients and the cholesterol in that fat you might not show a change in the total amount of labile cholesterol before and after thyroid administration This is suggested because one of the first effects of thyroid administration is to alter drastically the skin s appearance as well as the fact that the effects of hypercholesterolemia in animals are very evident in the skin follicles The skin in short as well as the subcutaneous fat is a marvelous reservoir for cholesterol I think there might be enough cholesterol and cholesterol mobility there to upset the balance to the point that your data could be interpreted as showing that the thyroid really does not stimulate synthesis

GOULD In these experiments in which blood samples are taken 4 hours after administration of acetate the skin cholesterol could not have very much effect because of the fact that skin cholesterol does not interchange with plasma cholesterol very rapidly

FREEDBERG I have some data on the specific activity of the skin after the intra

venous infusion of C^{14} labeled cholesterol (4 C^{14}) in a patient who unfortunately died 15 days after we gave him the material intravenously. The specific activity in the skin was 50% of that of the plasma and liver which were in equilibrium at that time. The aorta incidentally also had a lower specific activity than the plasma.

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shortly thereafter as in dogs liver free and plasma free cholesterol come to equilibrium in a matter of 2 hours or so and then remain equal within experimental error

FREEDBERG With reference to thyroxine metabolism in hypogonadism and hypometabolism Dr Kurland and I have studied three such patients The thyroxine degradation rate was slow in these patients In one of the patients we administered testosterone a fairly large dose and the curve of disappearance suddenly increased within the first 24 hours as if there were a washing out of the thyroxine from the blood stream but in the subsequent 48 to 72 hours the curve of disappearance approximated that seen before we gave the testosterone We interpreted this as representing a change in the volume of distribution but not in metabolism following testosterone

WHITE I wonder if I might address three points or questions to Dr Gould In referring to species differences I believe you made the statement that in general we know that the human is more sensitive to the loss of thyroid function than are other animals What did you mean in terms of quantitative data? Secondly perhaps I am reading more into your statements than you actually meant I don t think you were intimating that the differences in the *in vivo* versus the *in vitro* rates of incorporation of acetate into cholesterol under various experimental conditions necessarily invalidated the *in vitro* approach particularly because the *in vitro* system became much slower Obviously *in vivo* you still have access to other energy sources which are necessary for the synthetic process whereas in the isolated slice you are running downhill pretty rapidly with respect to energy reserves Thirdly it would seem to me that while one can establish a correlation between liver cholesterol concentration in the liver and the rate of cholesterol synthesis in this organ this may be so only under circumstances in which a third variable is not entering the picture e g absence of a hormonal function as in the hypophysectomized or adrenalectomized animal It is well known that cholesterol synthesis is more rapid in the presence of an adequate supply of carbohydrate and it would seem to me that interpretation of data is difficult for the hypophysectomized or adrenalectomized rats since there exist marked variations in the supply of available energy factors under those circumstances

GOULD In answer to your first question my remark was intended to be a question not a statement of fact It is my impression that rats made hypothyroid by I^{131} are less sick than patients with full blown myxedema but I would like to hear from the endocrinologists present whether that is valid or not In answer to your second question I agree entirely with your statement about energy availability My point is that *in vitro* and *in vivo* methods may give different results so that both types of methods should be used to avoid being misled I don t think either approach is necessarily invalid or necessarily completely reliable It is puzzling that the addition of glucose to liver slices from fasted rats had no effect on the rate of cholesterol synthesis The effect of fasting is apparently more complex than merely the absence of liver glycogen

Now as to your third question the hypophysectomized animals shown at the bottom of the curve (Fig 4 H) were not only hypophysectomized but were fasted to make them as nearly comparable as possible with irradiated rats The increase in total liver cholesterol found under these conditions in adrenalectomized and in hypophysectomized rats suggests the possibility that there may be an increase in liver free cholesterol If this is so the decrease in the rate of synthesis might be due to this effect of hormone deficiency alone

I would like to ask Dr Popjak if he has any comments on the energy supply necessary for cholesterol synthesis in homogenates

POPJAK I don't think I have anything substantial to add because we have not studied homogenates derived from animals of different nutritional states Certainly in the course of our fractionating the homogenates we do remove the glycogen although I suppose with the microsomes a small amount of glycogen is added to the preparations but we do supply the energy in the form of ATP (adenosinetriphosphate) and any glycogen present there does nothing else but in the presence of glycolytic enzymes and ATP generates some glucose 6-phosphate and TPNH (reduced form of triphosphopyridine nucleotide)

BERGSTROM Knowledge as to the cholesterol concentration in the human liver and its relation to the rate of synthesis is certainly lacking in the human It does not seem to be generally appreciated how simply and safely 15 mg liver biopsies can be obtained by puncture from the back Dr Ekdahl will publish his thesis this spring in which he has made an extensive study of the bile acid conjugation in normals and in different cases of liver disease I believe this technique could be used more extensively in studying the cholesterol metabolism in man

A Comparison of the Participations of Fibroblasts and Reticulo endothelial Cells in the Synthesis and Metabolism of Cortisol and Cholesterol

Thomas F Dougherty and David L Berliner

Department of Anatomy University of Utah Salt Lake City Utah

For some years a major effort in our laboratory has been directed to studying the interrelationship of steroid hormone metabolism and the anti inflammatory and lymphocytolytic effects of these hormones These investigations have led us to believe that the primary sites of physiological effect and in addition the metabolism of steroid hor mones are related to the functions of reticuloendothelial and fibroblastic cells (9)

Briefly it has been found that cortisol tends to localize within or at the surface of fibroblasts (11 13) In studies of radioautographs of many tissues and cells it appears that the fibroblast is the main cellular site for localization of hydrocortisone On the other hand it has been shown that cortisol tends to destroy reticuloendothelial cells Other effects of cortisol on fibroblasts are that it tends to round them up increase their basophilia and increase intracellular reducing substances in the cytoplasm (11) It has also been shown that these rounded up fibroblasts are tougher i e they resist cellular destruction during the alterative stage of inflammation (13) We have postulated that in deed this is a mechanism by which cortisol inhibits inflammation i e that by increasing the resistance of these cells the chain reaction of cellular destruction which potentiates inflammation is interrupted (9 13 14) Others have amply demonstrated that cortisol interferes with other fibroblastic functions such as the formation of collagen fibers and the synthesis of ground substance (see review 9) It seems clear then that cortisol acting upon fibroblasts brings about certain changes in the function and appearance of these cells at a time when they are absorb ing and metabolizing this hormone Cells of the macrophage system on the other hand do not appear to take up cortisol and retain it for any great period of time

Recently we have also found that the length of time that cortisol is present within the fibroblast is much shorter than the period during which inflammation is reduced (12) It appears therefore that cortisol triggers some mechanism which in turn continues even after this hormone is catabolized and excreted Extensive studies concerning this point have been performed in order to elucidate the mechanisms by

which cortisol brings about alterations in fibroblasts which are related to the function of this hormone It is now known how long cortisol is localized by the fibroblast (12) and in addition we have shown that fibroblasts of loose connective tissue bring about transformations in the molecule of cortisol during the period of time that this hormone exerts its anti inflammatory potency (3) These transformations are given in detail elsewhere Briefly they might be characterized in general as oxidations and reductions of substituted positions on the gonane nucleus which ultimately end with removal of the side chain At the time that these transformations are occurring conjugates form in the liver (4) and result in removal of these hormones from the organism Thus the products of fibroblastic metabolism of cortisol are removed from the cellular site of their transformations and are eliminated This whole process is very rapid so that even in the human being transformations are found in the urine within 5 minutes after administration of radio active cortisol (2) Elsewhere we will discuss the meaning of the transformations of the cortisol molecule with respect to the mechanism of its anti inflammatory effectiveness

The fact that it is mainly the fibroblast which performs cortisol catabolism has been demonstrated also in our laboratory by incubating radioactive cortisol with fibroblasts in tissue culture (20) The transformations produced by pure cultures of fibroblasts are exactly the same as those found for fibroblasts of incubated loose connective tissue There is no doubt then that a major performer of cortisol metabolism at the cellular level is the fibroblast We have also demonstrated that cells of the macrophage and normal lymphocytic varieties cannot perform these transformations but in the process of cortisol action are destroyed (10) There is then a very distinct difference between the capacities of these cells to respond to the action of cortisol

Other steroid hormones have also been studied and the results of these investigations will be presented at another time One aspect of arteriosclerosis research is that many individuals interested in the etiology of such diseases concern themselves with cholesterol deposition as though it occurred without the participation of cells Attention should be called to the fact that it has long been known that the formation of atheroma most likely begins intracellularly (reviewed by Altschul 1) and it has frequently been a subject of speculation that cholesterol deposits because the cells which take it up retain it are later destroyed and intracellular cholesterol is left in place Gradually deposits of this sterol are embedded in ground substance and reticular fibers which are produced by connective tissue cells Since we had a considerable amount of experience in our laboratory with the participation of fibro

blasts and macrophages in the metabolism of steroids it seemed worth while to compare the roles of these cells in cholesterol metabolism A comparison of the way fibroblasts and macrophages metabolize cholesterol is particularly worthwhile since these are precisely the cells which are concerned to the greatest extent with steroid and cholesterol metabolism Consequently methods which had been applied to a study of cortisol metabolism were applied to that of cholesterol

SYNTHESIS OF CHOLESTEROL FROM ACETATE BY TISSUE CULTURES OF HUMAN FIBROBLAST

Fibroblasts derived from human myometrium and cultured for about 3 years in medium #79 (5% chick embryo extract 20% normal horse serum and 75% solution #79) as described by Swim and Parker (21) were supplemented with 2 μc of acetate 2 C^{14} per 12 ml of fluid Twelve ml of fluid were used in each incubation flask The specific activity of the acetate was 1 mc/mmole The procedure of the incubation will be given in detail elsewhere In general it was the usual methodology employed for such studies T60 flasks were set up with approximately 6 × 10 cells in #79 solution containing acetate 2 C^{14} medium along with the appropriate number of small flasks to be used in determining the degree of cellular proliferation The tubes were inclined at an angle of 5 and incubated overnight at 37 C in a stationary position and then were placed in a roller apparatus at 37 C The medium was changed on the 4th day and pooled On the 8th day the medium from the flasks was pooled again and the cells were harvested and washed twice with Earls saline The Earls saline was added to the 8th day sample At the time of innoculum there were 5 4 × 10 cells at the 4 day interval the number of cells was 2 1 × 10 At the 8th day there were 5 8 × 10^6 cells

Extraction and identification of cholesterol were done as follows the cells and the medium were extracted separately but the same extraction procedure was used for both The samples were saponified and the resulting mixture extracted 3 times with chloroform The combined chloroform extract was evaporated and the residue was directly chromatographed on paper using a modification of the hexane system of Zaffaroni (22) in which formamide is not applied to the paper but the sample is placed directly on the paper and run in the hexane mobile phase Under these conditions cholesterol has an R_f of 0 65 The chromatographic zone corresponding to cholesterol was eluted Nonradioactive carrier cholesterol was added to the eluate and the cholesterol was precipitated with digitonin The digitonin complex was hydrolyzed and three separate crystallizations were performed on

which cortisol brings about alterations in fibroblasts which are related to the function of this hormone It is now known how long cortisol is localized by the fibroblast (12) and in addition we have shown that fibroblasts of loose connective tissue bring about transformations in the molecule of cortisol during the period of time that this hormone exerts its anti inflammatory potency (3) These transformations are given in detail elsewhere Briefly they might be characterized in general as oxidations and reductions of substituted positions on the gonane nucleus which ultimately end with removal of the side chain At the time that these transformations are occurring conjugates form in the liver (4) and result in removal of these hormones from the organism Thus the products of fibroblastic metabolism of cortisol are removed from the cellular site of their transformations and are eliminated This whole process is very rapid so that even in the human being transformations are found in the urine within 5 minutes after administration of radio active cortisol (2) Elsewhere we will discuss the meaning of the transformations of the cortisol molecule with respect to the mechanism of its anti inflammatory effectiveness

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cholesterol Constant specific activity was maintained at 1100 counts per min /mg The last crystallization sample was divided into two ali quots The first one was oxidized to cholestene 3 one and again crystal lized *to constant specific activity*

The specific activity was 1120 counts per min /mg The second aliquot was acetylated forming cholesterol acetate which on crystal lization gave a specific activity of 1128 counts per min /mg These two figures for S A include the correction for the molecular weight change of the derivatives Figure 1 summarizes the procedure which

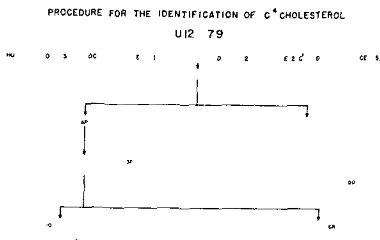


FIG 1 Scheme of the procedure used in the identification of C^{14} cholesterol isolated from incubations of acetate 2 C^{14} with tissue cultures of fibroblasts

was used A similar result was obtained for the extraction of the cells Both the fluid medium and the cells yielded radioactive cholesterol *The total per cent conversion from acetate to cholesterol was in the* range of 0 5–0 8% This percentage of conversion is that portion of the acetate originally added which became incorporated into cholesterol molecules It is not the portion of total carbons of cholesterol formed

THE STORAGE OF CHOLESTEROL 4 C^{14} IN THE RETICULOENDOTHELIAL SYSTEM

Forty intact animals were injected intravenously with 1 μc of cho lesterol 4 C^{14} dissolved in either 0 1 ml propylene glycol and alcohol or in 0 3 ml horse serum These rats were divided into three groups (*1*) control (*2*) thyroxine treated (25 μg) and (*3*) ACTH treated (1 international unit of ACTH having 20 international units/mg) The rats were sacrificed at various intervals from 1 hour to 12 days as is

shown in Fig 2 Another series of rats were eviscerated as described by Ingle (15) and injected intravenously with cholesterol-4 C^{14} Thus the experiments included intact animals intact given ACTH noneviscerated adrenalectomized given ACTH and eviscerated nonadrenalectomized with ACTH (Table I) A variety of tissues and organs were studied which are given in Table I The relative activity expressed in CPM/mg of dry tissue was studied (5) The tissues were homogenized and directly plated on aluminum plates Correction for self absorption of *beta* rays was applied in each case

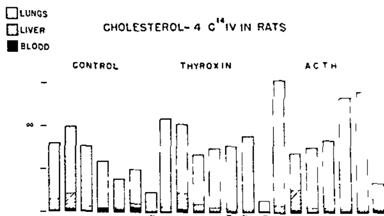


FIG 2 Comparison of the relative specific activities based on dry weight of tissue of control thyroxine treated and ACTH treated rats given cholesterol-4 C^{14} intravenously The animals were sacrificed at various intervals over a 12 day period

In order to compare the metabolism of cholesterol to cortisol 20 mice were injected in the same way as the rats with cortisol-4 C^{14} and as above the organs and tissues were removed at various intervals as shown in Fig 3 and the relative activity of these tissues was determined In Figs 2 and 3 we have portrayed the CPM/mg of blood lungs and livers In Fig 2 it may be seen that in every case studied the lungs had a higher relative activity than any other organ The liver was second and finally the blood There was a rapid increase in radioactivity in the lungs of all groups which decreased slowly with time In the thyroxine treated group there seemed to be a slightly higher but not significantly different concentration of cholesterol in the lung than in the control However the ACTH treated noneviscerated rats had a markedly high activity in their lungs as compared to any other of treated or nontreated animals Later at 6 hours there was a decrease but still later the lungs of these animals were higher in activity and re

maintained so as long as 12 days. In comparison the cortisol treated mice had the highest radioactivity in the liver, then the lung and finally the blood. That is to say, there was a specificity in the trapping of one steroid in relation to another by different organs. The gonane nucleus of both cortisol and cholesterol is not metabolized or destroyed by the organism (12, 19). Only the side chain of cholesterol or the substituted groups or side chain of cortisol are changed (8, 12). Cortisol has a very short half life compared to cholesterol. Cholesterol is retained in the rat for about 15 days (19). However, cortisol has a half life in rats, humans or mice of about 50 to 60 minutes (2, 12). We know from previous studies that the liver is the main or only organ which is capable of conjugating cortisol (4) so that this hormone may be excreted mainly in the urine. However, cholesterol is a non- or very poorly metabolized sterol and is retained for a long time. It is interesting to notice from Table I that in the intact adrenalectomized animal treated with ACTH there was a higher concentration of cholesterol in the lung than in the intact nontreated animal. Thus ACTH apparently has an effect on the concentration of cholesterol in the absence of the adrenal. In Table I it may also be noted that the relative activity from the intact, intact and ACTH treated, and eviscerated and ACTH treated animal showed that there was an increase of cholesterol in the adrenal which was higher in the eviscerated ACTH treated animals.

TABLE I
DISTRIBUTION OF RADIOACTIVITY (CPM/MG DRY TISSUE) 45 MINUTES AFTER INFUSION OF CHOLESTEROL-4-C^{14}

Treatment	Intact	Intact ACTH	Noneviscerated adrenex + ACTH	Eviscerated given ACTH
Lung	186.7	501.0	665.0	560.0
Liver	4.5	10.9	9.4	—
Spleen	7.4	5.3	6.4	—
Blood	1.1	3.2	1.0	3.5
Adrenal	1.9	3.6	—	8.0
Aorta	0	1.4	1.5[a]	3.4
Pituitary	2.5	1.8	1.6[a]	4.4
Kidney	0	0	0	1.3
Heart	0	0	1.0	1.4
Muscle	0	0	0	0
Small Intestine	2.1	1.3	2.6	0
Testis	0	0	0	1.0
Thymus	0	0	0	1.7
Fat	0	0	1.6	0
Brain	0	0	0	0

[a] Insignificant count

The cellular site of cholesterol deposition in the lung is in the reticulo endothelial cells of this organ. This is shown by radioautography of the 4 C^{14} cholesterol treated lungs (Figs 4 and 5). The epithelial cells of the bronchi do not contain C^{14}. The macrophages which contain most of the labeled cholesterol are in the alveolar connective tissue and septal cells. The greatest radioactivity was found in the region of the respiratory bronchioles.

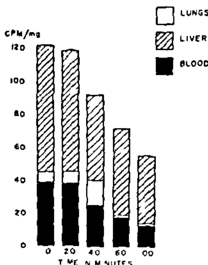


FIG 3 Comparison of the relative specific activities (dry weight) of tissues of rats given cortisol-4 C^{14} intravenously and sacrificed at intervals up to 100 minutes

DISCUSSION

Roger and Binet in 1925 (17) suggested that the lung was the major site of cholesterol storage in the body. In 1931 Seeman (18) performed a series of studies on cholesterol metabolism and came to a similar conclusion. In addition this investigator administered cholesterol into a peripheral artery and into the portal vein and found that even when *it was administered by this route and therefore had to pass through* the liver before the lung its primary site of deposit was still in the lung. Cioni (7) stated that the cholesterol in the lung is in the reticulo endothelial cells of the alveolar wall. Quensel (16) came to similar conclusions and suggested that the lung played a major role in the removal of cholesterol from the body by way of the phagocytes in the lung which he thought would then move out in the sputum. More

recently it has been shown by Brigdon and Gordon (6) that unesterified fatty acids are concentrated in the lung to a greater extent than any other organ 200 minutes after they were administered It would seem that fluctuations in cholesterol content of the lung reflect generalized changes in reticuloendothelial uptake of this substance It also seems possible but to us less likely that fibroblasts which form cholesterol could retain it and upon their disintegration release it Since both

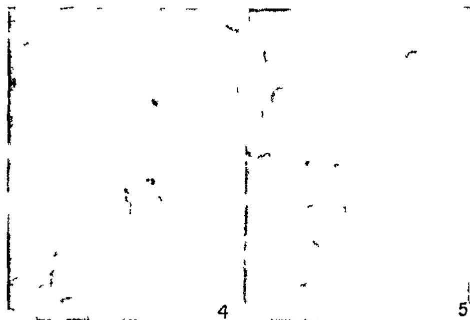


FIG 4 Radioautograph of reticuloendothelial cell The black dots coming from this alveolar macrophage are beta ray tracks from the radioactive cholesterol Magnification 720×

FIG 5 Radioautograph of lung epithelial cells which do not contain any radio active material Note the beta tracks from the macrophage adjacent to the epithelial cells Magnification 720×

fibroblasts and macrophages are ubiquitous it does not seem necessary to postulate that the same cell which synthesizes cholesterol must necessarily store it and thus subsequently initiate extracellular de position

ACKNOWLEDGMENTS

The work reported here related to the production of cholesterol by fibroblasts in tissue cultures was done in collaboration with Dr H E Swim of the Department of Microbiology Western Reserve University who performed the tissue culture portions of this experiment

The radioautographs were made by Dr W S S Jee of the Department of Anatomy and Division of Radiobiology University of Utah College of Medicine This was supported by a grant from the United States Army (DA-49 007 MD 130) Department of the Army Committee on Stress

Details of the tissue culture methods and radioautograph methods will be given in separate publications which will deal with these matters exclusively

The authors also wish to thank Dr Robert T Hill Executive Secretary Endocrinology Study Section National Institutes of Health Bethesda Maryland for his generous contributions of cortisol-4 C^{14} thus permitting this research

References

1 Altschul R Selected Studies on Arteriosclerosis C C Thomas Springfield Illinois (1950)
2 Berliner D L and Berliner M L *Federation Proc* **16** 153 (1957)
3 Berliner D L and Dougherty T F *Proc Soc Exptl Biol Med* **98** 3 (1958)
4 Berliner D L Grosser B I and Dougherty T F *Arch Biochem Biophys* (in press)
5 Berliner D L and Wiest W G *J Biol Chem* **221** 449 (1956)
6 Bragdon J H and Cordon R S Jr *J Clin Invest* **37** 574 (1958)
7 Cioni C *Sperimentale* **86** 587 (1932)
8 Dorfman R I *in* The Hormones" (G Pincus and K V Thimann eds) Vol III p 589 Academic Press New York 1955
9 Dougherty T F *in* Progress in Allergy Vol IV p 319 Little Brown & Co Boston 1955
10 Dougherty T F *in* The Leukemias Etiology Pathophysiology and Treatment p 293 Academic Press New York 1957
11 Dougherty T F Bigler R Schneebeli G L and Salhanick H A *Ann N Y Acad Sci* **64** 466 (1956)
12 Dougherty T F Brown H E and Berliner D L *Endocrinology* **62** 455 (1958)
13 Dougherty T F and Schneebeli G L *Ann N Y Acad Sci* **61** 328 (1955)
14 Eyring H and Dougherty T F *Am Scientist* **43** 457 (1955)
15 Ingle D J *Exp Med Surg* **7** 34 (1949)
16 Quensel U *Upsala Laek foer* **38** 1 (1932)
17 Roger G H and Binet L *Rev méd (Paris)* **42** 1 (1925)
18 Seemann C *Beitr pathol Anat u allegem Pathol* **83** 705 (1930)
19 Siperstein M D and Chaikoff I L *Federation Proc* **14** 767 (1955)
20 Sweat M L Grosser B I Berliner D L Swim H E Nalbors C J Jr and Dougherty T F *Biochim et Biophys Acta* **28** 591 (1958)
21 Swim H E and Parker R F *Am J Hyg* **66** 235 (1957)
22 Zaffaroni A *Recent Progr in Hormone Research* **8** 51 (1953)

Discussion

HELLMAN I would like to ask Dr Dougherty in which form he injected the C^{14} cholesterol used in these experiments and whether he has any comparable data on cholesterol fed to animals after which he examined their tissues?

DOUGHERTY The cholesterol was given in propylene glycol One feeding experiment was performed which gave essentially similar results although the difference between lung and liver was not so great This work is being continued

HELLMAN The reason I asked the question is that administering cholesterol in this form is not giving it in true solution and actually you have a suspension of different particle sizes of radioactive cholesterol and the ability of various tissues to trap the cholesterol would unquestionably be a function of the particle size of the cholesterol injected These injection solutions age rapidly—they start out as very small particles and as they stand around just for a few hours the particles conglomerate and become much larger and I would suspect that one might wish to look into the effect of the particle size in the injection solution as it affects the distribution of the injected cholesterol

DOUGHERTY Dr Berliner I think should answer that question

BERLINER You will notice from one of the slides that we injected cortisol and cholesterol through the same route However cortisol was trapped mainly by the liver but with cholesterol the lungs retained mainly this radioactive sterol Therefore I don t think that crystallization of cholesterol in blood would be the cause of the trapping If this were the case then cortisol should have been also trapped by the lung

DOUGHERTY We did not find any radioactivity in the capillaries as shown by the radioautographs therefore there is no sign of embolism

KATZ I would like to ask the pathologists present what the lung lesions are in xanthomatosis When there is a high level of blood cholesterol which kind of lung cell has the cholesterol in the xanthoma? I think that Dr Dougherty dealt with the injections perhaps of particular matter as was pointed out by Dr Hellman Surely in the diseases that we are interested in we do not have the same condition—the material enters the blood in a different fashion

WILHELMI Would any pathologist like to comment?

HOLMAN As to the question about the *usual* findings in the lung there are various types of storage diseases including xanthomatosis It is not common to find foam cells within the pulmonary capillaries or in any of the reticuloendothelial cells of the lung in these conditions I am aware that such foam cell collections have been described and I have seen them on occasion but they are not the usual finding You were dealing with the rabbit in your experiments Dr Dougherty?

DOUGHERTY This was rat and I beg to differ with you on this ground that if you look at Jaffe s chapter on xanthomatosis cholesterolosis—and he was probably in his day one of the deans of pathology—you will see the lung lesions described and in fact Jaffe made quite a point of them Now this still does not mean that the lung must be involved in every type of cholesterolosis but as far as the nature of the cells is concerned they are reticuloendothelial cells I don t think cholesterol really goes to any other place that is administered cholesterol except to these cells It looks like a phagocyte process

FLOREY There is possibly something significant in this connection done by French and Morris in my department The work is not on cholesterol itself but it is connected with the disposal of particles If you take physiological fat particles that is to say chylomicra collected from rat lymph and inject them intravenously or into a perfused liver they are taken out almost exclusively by the liver They are taken out by the parenchymal cells and not by the reticuloendothelium If however an artificial emulsion of fatty particles is used the particles are taken out in the lung in the spleen and in the Kupffer cells of the liver and very little goes immediately into the liver cells These observations refer to chylomicra and not specifically to cholesterol This difference in distribution depends on whether a particle is of physiological origin or whether it is an artifact

BYERS We accumulated evidence concerning the location of fed cholesterol and of injected chylomicron cholesterol as being in the hepatic reticuloendothelial cells We believe the participation of phagocytic cells to be required for the normal expeditious disposal of fed cholesterol It is not generally realized that C^{14} cholesterol in lymph chylomicra has been found to leave the blood stream within 5 minutes of its entry only to reappear in the blood as soluble lipoprotein cholesterol after another 10 minutes (see Max Biggs in "Advances in Biology and Medical Physics" Volume V 1957) So rapid a removal rate is entirely consistent with the known rates of removal of particles by the reticuloendothelial system Indeed the curve of disappearance of plasma chylomicronous cholesterol flattens out immediately upon intravenous injection of foreign colloid and resumes its former slope immediately after the disappearance of the foreign colloid (see Neveu *et al* in the *Am J Physiol* November 1956) After oral cholesterol we have demonstrated excess fat and cholesterol in both isolated R E cells and R E cells *in situ* After intravenous injection of soluble lipoprotein cholesterol however excess cholesterol was found largely in hepatic parenchymal cells and very little in R E cells (see Friedman and Byers *Circulation* 10 491 1954)

CHAPTER 9

Influence of Hormones on Lipid Biosynthesis in Liver[1]

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This paper is a brief report of a portion of studies currently in progress on two problems the influence of the endocrine glands on lipid biosynthesis by the liver and humoral relationships between the liver and the peripheral tissues The data to be presented are concerned only with the extent of *in vitro* incorporation of radioactivity from acetate 1 C^{14} into the total digitonin precipitable sterols of rat liver slices Liver tissue has been obtained from normal fed and fasted animals of both sexes from castrated male and female rats and from castrated male and female rats treated with either testosterone propionate or estradiol respectively

EXPERIMENTAL

Rats of the Sprague Dawley strain weighing 250–350 g were killed by decapitation the livers excised and placed in cold Krebs Henseleit bicarbonate buffer pH 7 4 One gram of liver slices was analyzed for total cholesterol without incubation Other aliquots (250 mg each) of the liver slices were incubated in 4 5 ml of the Krebs Henseleit buffer to which was added 0 5 ml of 0 001 *M* sodium acetate 1 C^{14} Incubations were conducted with shaking for 3 hours at 37 C in small beakers placed in a Dubnoff incubator the gas phase was 95% O_2–5% CO At the end of the incubation the contents of 4 beakers representing 1 g of liver slices were combined and saponified by addition of 2 ml 15% KOH in 95% ethyl alcohol and boiling under reflux for 6 hours After cooling 20 ml of 70% ethyl alcohol were added and the nonsaponifiable lipids extracted four times with 20 ml portions of hexane The combined hexane extracts were dried over anhydrous Na SO_4 filtered the hexane removed *in vacuo* on a steam bath and the residue dissolved in 10 ml of acetone absolute ethyl alcohol mixture (1 1) One aliquot was used for the colorimetric determination of cholesterol by the method of Sperry and Webb (1) Two aliquots were each used for the prepara

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The paper was presented at the conference by Abraham White

Obtained from the Holtzman Co Madison Wisconsin

TABLE I[a]

INCORPORATION OF RADIOACTIVITY FROM SODIUM ACETATE 1 C^{14} INTO THE TOTAL DIGITONIN PRECIPITABLE STEROLS OF RAT LIVER SLICES

Type of *rat used*	No of *animals*	Body weight[b] (*g*)	Mean total cholesterol[c] (*mg/g*)	% of NaC H_3O 1 C^{14} counts incorporated into digitonin precipitable sterols	*P* values[d]
Male fed	17	302 (264–357)	1 7 ± 0 05	2 1 ± 0 2	
Male fasted	9	287 (260–312)	2 4 ± 0 11	0 23 ± 0 06	< 0 001
Male castrated studied 4 weeks postoperative	12	313 (285–334)	1 6 ± 0 07	2 4 ± 0 3	< 0 5
Male castrated 4 weeks postoperative injected with T P[e]	8	325 (305–364)	1 5 ± 0 10	1 5 ± 0 1	< 0 025
Female fed	9	271 (241–286)	1 8 ± 0 07	4 5 ± 0 8	< 0 01
Female castrated studied 4 weeks postoperative	6	294 (277–318)	1 6 ± 0 10	0 7 ± 0 1	< 0 001

[a] The data in this table were obtained during the period April to October 1957

[b] Range of body weights shown in parentheses

[c] Means and standard errors

[d] Values calculated for P in comparison with normal fed male rats except value for castrated female rats which is calculated for the difference from normal female rats

0 5 mg testosterone propionate subcutaneously daily for 14 days

TABLE II[a]

INCORPORATION OF RADIOACTIVITY FROM SODIUM ACETATE 1 C^{14} INTO THE TOTAL DIGITONIN PRECIPITABLE STEROLS OF RAT LIVER SLICES

Type of rat used	No of animals	Body weight[b] (g)	Mean total cholesterol (mg/g)	% of NaC H_3O 1 C^{14} counts incorporated into digitonin precipitable sterols[c]	P" values[d]
Male fed	6	363 (333–387)[d]	1 7 ± 0 11	0 9 ± 0 1	
Female fed	6	280 (255–312)	1 8 ± 0 03	1 9 ± 0 2	< 0 001
Female castrated 6 weeks postoperative injected with sesame oil[e]	6	323 (287–347)	1 7 ± 0 09	1 9 ± 0 3	Not significant
Female castrated 6 weeks postoperative injected with estradiol[f]	3	255 (244–273)	1 7 ± 0 04	2 2 ± 0 5	Not significant

[a] The data in this table were obtained during the period October 1957 to February 1958
[b] Range of body weights shown in parentheses
[c] Means and standard errors
[d] Values calculated for P in comparison with normal fed male rats
0 1 ml subcutaneously daily for 4 days
[f] 0 1 mg estradiol benzoate in 0 1 ml sesame oil subcutaneously daily for 4 days

tion of total digitonin precipitable sterols. The precipitated digitonides were filtered onto a weighed planchet and counted in a gas flow counter with a thin end window. Counts are corrected to infinite thinness.

Testosterone propionate and estradiol benzoate were kindly supplied by the Schering Corporation, Bloomfield, New Jersey.

RESULTS

The data which have been obtained are presented in Tables I and II. In Table I are data for animals studied during the period April to October 1957, while the data in Table II have been obtained during the period October 1957 to February 1958. The data are divided in this manner because of the differences between the results obtained in the two periods mentioned. In the initial experiments, the extent of incorporation of radioactivity from acetate-1-C^{14} into digitonin precipitable sterols by rat liver slices was significantly greater (approximately 2–3 times, Table I) than that seen in the subsequent studies (Table II). The lower values in Table II have been consistently observed in data obtained since that time, and there is as yet no explanation for the earlier higher incorporation values. The lower values observed as a consequence of fasting the animals prior to sacrifice are a well established phenomenon (2).

It will be seen that in either group of data, liver slices from normal fed female rats incorporate approximately twice as much total radioactivity from acetate-1-C^{14} into the digitonin precipitable sterols as do liver slices from normal fed male rats. Liver slices obtained from female rats 4 weeks after castration show a significant reduction in their extent of incorporation (Table I). In contrast, castration of the male rat or treatment of the castrated animal with testosterone propionate does not alter the capacity of liver slices to incorporate radioactivity of acetate-1-C^{14} into the digitonin precipitable sterol fraction. On the other hand, the data in Table II suggest that castration of the female or treatment of the latter with estradiol has not, in the later series of experiments, altered significantly the extent of incorporation from that seen with liver slices from normal female rats. These data are in contrast to those of Table I.

Thus the data are at present difficult of interpretation. The animals represented by the data in Tables I and II were housed, fed, and handled in identical manners.

It may be mentioned in closing that administration of hydrocortisone to normal male or female rats appears to depress incorporation *in vitro* of radioactivity from sodium acetate-1-C^{14} into the digitonin precipitable sterols of liver slices from these animals. This is strikingly the case if

the animals lost weight during hormonal treatment although it has also been seen in experiments in which the amount of hydrocortisone injected did not cause weight loss

REFERENCES

1 Sperry W M and Webb M *J Biol Chem* 187 97 (1950)
2 Tomkins G M and Chaikoff I L *J Biol Chem* 196 569 (1952)

DISCUSSION

BOYD If I could show two slides to illustrate the point that the rate of cholesterol synthesis in the liver the serum cholesterol level and the liver cholesterol levels seem to be related in a complex manner We have performed experiments in which we studied the serum cholesterol of castrated male and female rats Now in Dr White s experience the rate of liver cholesterol synthesis of the male castrated animals is only slightly above that of the intact animals and we find that castration in the male rat has little influence on the serum cholesterol level (+10%) In the female Dr White has shown that there is marked depression of hepatic cholesterol synthesis after castration while we have found that in castrated females the serum cholesterol level is markedly increased (+30%)

ROSENFELD Dr White in the second part of your experiment when the per cent counts incorporated were decreased in your animals was the measurement of the total cholesterol the same as you have previously measured?

WHITE It was

ROSENFELD I wonder if you could comment about the stability of the acetate which you used Do you have any information on that?

WHITE We have checked this Dr Rosenfeld I would like to think that we have considered every one of the more obvious variables Something occurred rather abruptly in our studies to the activity of the incorporating system The sex difference still obtains but the incorporating activity of the system has declined very markedly We have been totally at a loss to understand this and we have checked many things except the season of the year

KENDALL It seems to me that you have controll d everything except for the length of daylight It has been shown that in various species of animals particularly birds a great many physiological functions can be related to changes in length of day Maybe you are demonstrating it in rats

WILHELMI May I interject at this point? Dr Pickford who keeps minnows in a wine cellar in an old house in New Haven for the purpose of studying these creatures has noticed that the normal fish who are now outside of the apparent influence of the seasons still come into season at the proper time of year although they are in stable environmental conditions comparable to those of your rats So I think it doesn t necessarily follow that the artificial maintenance of the steady environment eliminates these effects

WHITE As the spring season will soon be upon us we should know

PICK I may not have heard it but as the age and sexual maturity comparable in the two series?

WHITE The age was comparable whether the sexual maturity was comparable I am not certain This was not studied

FURMAN I was just wondering if Dr White has checked the rat chow for possible contamination with estrogen

WHITE No

DRILL I was going to comment on the same thing that different batches of diet can throw off our estrogen assays and I think perhaps you Dr Pincus have also noticed the same thing So we have shifted over to a synthetic diet for such studies

WERTHESSEN On this sex difference Dr White is this really more biogenesis or is it more turnover in those livers? Do you measure the amount of cholesterol before and after incubation? If not and if it cannot be I would like to suggest that you use the aorta of these animals There is work published by Eisley and Pritham which indicates that you can measure real changes in cholesterol *concentration* in comparable preparations of the aorta *in vitro*

WHITE I think perhaps the word biogenesis is correct since new cholesterol molecules are being formed We are not inferring net synthesis We are measuring incorporation of isotope into cholesterol this is probably more closely related to turnover of cholesterol The term digitonin precipitable sterols" is preferred to cholesterol or to quote Dr Schwenk "cholesterol and its companions"

STAMLER Dr White mentioned that the hydrocortisone treated rat manifested an increase in liver cholesterol concentration and a concomitant depression of cholesterol synthesis Are there any observations relating to lipemia in these rats and to the time course of these events? I pose this question because in the chick hydrocortisone induced a marked hyperlipemia persisting with continued administration for several weeks Particularly during the early phases of this phenomenon tracer studies carried out in cooperation with Gordon Gould indicated a considerable increase in synthetic rate of cholesterol from labeled acetate

WHITE We did not measure the level of serum lipids The inhibition of incorporation can be seen with a dose of hydrocortisone which does not cause a loss in body weight It is very easy to depress incorporation of acetate into cholesterol in liver slices of animals by merely producing a depression in body weight If you give enough hydrocortisone or another substance which restricts food intake and produces a loss of body weight you can show this phenomenon But these animals did not lose weight

POPJÁK I think that some of my observations made on the liver of pregnant rabbits during the second part of pregnancy may be relevant to some of Dr White's results During the second half of pregnancy there is an extraordinary depression of cholesterol synthesis I have never seen anything like it It is about 1/100 of the normal and at the same time these animals develop very very marked hypocholesterolemia Their serum cholesterol values decline to 5–10 mg % I don't know the hormonal pattern in rabbits but I believe that during the second half of the term their progesterone predominates and I wonder if anyone has studied the effects of progesterone on cholesterol synthesis

MILCH Dr White pointed out that the terms cholesterol biogenesis" or "synthesis were not strictly correct Would you say Dr White that the term incorporation" is strictly correct?

WHITE I said "incorporation" was a more correct term although new cholesterol molecules are being biosynthesized

MILCH Your data then fail to demonstrate a difference of cholesterol *synthesis* between male and female but do demonstrate a difference of acetate C^{14} incorporation It may very well be that the female has a larger acetate pool

WHITE It is possible

GOULD In liver slice studies the size of the acetate pool can be both deter

mined and maintained essentially constant by adding enough acetate so that the dilution due to the endogenous acetate present in the liver or produced during incubation is relatively unimportant We use 5–10 mg of acetate per gram of liver slices routinely and have not encountered inhibitory or toxic effects at this level The acetate concentration is about 0 005–0 01 M If the results are expressed in terms of per cent of the C^{14} incorporated they may of course depend on how much C^{14} was used as well as on how rapid the cholesterol biosynthetic rate was We believe it is preferable to express the synthetic rate in terms of micromoles of acetate converted into cholesterol per gram of liver slices Enough acetate must be present in the medium in all experiments so that it constitutes an infinite reservoir and it must have a constant specific activity Then one can really measure the capacity of the liver tissue to synthesize cholesterol in other words the rate of synthesis not merely the amount of incorporation This is not strictly speaking "turnover" because the rate at which cholesterol is being broken down is not known If one uses only trace amounts of acetate C^{14} the size of the endogenous acetate pool will be a factor and the results will be difficult to interpret

WHITE Yes Dr Gould there is a relationship of acetate concentration to degree of incorporation into cholesterol One of the problems that one runs into is that higher concentrations of acetate actually seem to inhibit incorporation and the only way practically to get around this is to use acetate with a higher specific activity We have started with approximately 150 000 to 300 000 counts in the form of 0 001 M acetate

MILCH With respect to Dr Gould s point that the addition of sufficient acetate would obviate the necessity for identifying a difference between cholesterol synthesis and acetate incorporation we must consider just how the experiment is performed In most instances weightless counts of acetate are actually added and thus one fails to influence the size of the acetate pool Even if such low specific activity of acetate were included so as to equalize acetate pool size we would still be faced with the problem of acetate inhibition that Dr White pointed out in addition to the fact that we are not sure as to just what the acetate pool configuration is immediately prior to incorporation It seems that this entire matter of "synthesis" and incorporation has been too long loosely considered synonymous and when one attempts to be precise he finds that results obtained by the *in vitro* approach are almost universally indicted

CHAPTER 10

The Arterial Wall as an Organ[1]

RUSSELL L HOLMAN HENRY C MCGILL JR JACK P STRONG
JACK C GEER AND MARION A GUIDRY

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Much of the current research work and literature on atherosclerosis is formulated on the proposition that too much of something in the diet leads to too much of something in the blood and this in turn leads to too much of something in the blood vessel wall These postulates imply that filtration is the method whereby the noxious excess accumulates in the blood vessel wall The purpose of this presentation is to review some of the existing data which are difficult to reconcile with this filtration hypothesis and to emphasize other data which indicate that the arterial wall is something more than a passive filter and that it is indeed an organ in every sense of the word

DATA DIFFICULT TO RECONCILE WITH FILTRATION

On the *theoretical* side the similarity in the chemical composition of the lipids on the two sides of the endothelial barrier in the earliest stages of atherosclerosis would seem to support filtration My physicist friends tell me however that this very similarity constitutes strong evidence against filtration and that molecules of such diversified size shape electrical charge chemical composition and configuration such as cholesterol cholesterol esters phospholipids and triglycerides could not be expected to "filter at a uniform rate which would be necessary to account for the similarity in relative proportions of these lipids in the early lesions (fatty streaks) and in the blood On the other hand easily filtrable building units such as acetyl CoA could be fabricated by the cells of various organs including the arterial wall into common end products on both sides of the endothelial membrane under the influence of various hormonal and enzymic systems

On the more *immediate side* the appearance of the lesions their size shape and distribution in both man and experimental animals does not suggest filtration as the method of their formation The spotty unpredictable size and shape of the lesion including at times very sharp outlines of individual lesions are difficult to explain by any mechanism but to the author forces that imply dynamic movement under hormonal

[1] Supported by grants from the National Heart Institute

and enzymic as well as physical and chemical control render some of these difficulties less incongruous than those that imply passive in filtration

Further chemical analyses of normal and sudanophilic areas in the arterial wall have shown a consistent increase in cholesterol and other lipids in the sudanophilic areas in any given aorta but have failed to reveal a critical level of lipid concentration above which sudanophilia is constantly present and below which sudanophilia is constantly absent Furthermore the average concentration of one of these lipids (cholesterol) in the normal (nonsudanophilic) aorta in the Negro race in the age group from 11–15 years was 2 3 times as great as in the aorta of the white race in the same age group Thus analyses of uninvolved normal areas in some aortas have yielded a higher concentration of cholesterol than involved (sudanophilic) areas in other aortas (2) Hence local factors other than cholesterol concentration (and probably that of other lipids) must determine the presence or absence of lesions and some metabolic gradient must be involved in this determination

On the *experimental* side there are two masses of data that have accumulated over the decades that just do not fit in with the concepts of filtration The first of these has to do with the prolonged period of cholesterol feeding necessary to induce atherosclerotic lesions in rabbits chickens hypothyroid dogs or in whatever species and under whatever conditions one elects Despite the fact that the blood cholesterol level begins to rise in the first week or two of cholesterol feeding lipid infiltrations in the arterial wall are rarely demonstrable before the third month of such feeding and with some species of animals many more months of cholesterol ingestion and sustained hypercholesterolemia are required before predictable lesions make their appearance Furthermore there is no strict correlation between the degree of rise in blood cholesterol level and the degree of lesions While it can be stated that some elevation in blood cholesterol level is usually prerequisite for lesions everyone who has worked with animals is familiar with the occasional finding of animals with marked hypercholesterolemia that just do not have lesions

The second mass of data that has accumulated is even more damnable to the filtration hypothesis namely those data showing that certain hypercholesterolemias can be disassociated from atherosclerotic lesions Duff and McMillan (1) McGill and Holman (7) and others have failed to find atherosclerotic lesions in rabbits made diabetic with alloxan despite prolonged elevations of blood cholesterol levels Kellner (6) and others have produced similar or even more marked hypercholesterolemia in rabbits with intravenous injections of detergents such as Tween 80

and Triton A 20 without any resulting arterial lesions The same is true of the "lipid mobilizing factor" of Seifter (8) about which you will hear more later in this program

Thus there are theoretical experimental and human data that are difficult to reconcile with the filtration hypothesis implied in the sequence (*1*) "too much of something in the diet" (2) "too much of *something in the blood" and (3) "too much of something in the arterial* wall" This does not mean that diet and filtration are not involved in atherogenesis but it does mean that the direct application of this sequence is an oversimplification of the problem In the remainder of the time allocated to me I would like to call attention to existing data which indicate that the arterial wall is something more than a passive filter that it is indeed an organ in every sense of the word and that it plays an active role in atherogenesis

Dorland defines an organ as a part of the body having a specialized function The basic questions about the arterial wall are (*1*) How big is it? and (2) What does it do?

If for the moment we estimate very roughly the total weight of the various segments of the arterial wall as follows

1	Elastic arteries (aorta carotids and subclavians)	100 g
2	Muscular arteries	300 g
3	Arterioles	1000 g
4	Capillaries and basement membranes	3000 g

we can assign the percentages for the various components shown in Table I and end up with the total weight in grams shown in Table II

Even if the estimated proportions of the various tissues vary by as much as 50% and the total figures are as much as 2–10 times greater than reality it is obvious that we are dealing with a sizable organ possibly as large as the liver and bone marrow combined and approaching the order of magnitude of the blood itself This system of tubular units must be in a constant state of activity—both physical and chemical —for life itself depends upon it Furthermore the various component tissues of this tubular system must be in dynamic equilibrium with all the other tissues and organs of the body for it is this give and take between the various fluid and formed elements of the body that constitutes life

So much for the component tissues and estimated size of the organ What are its specialized functions? The transport of blood is an obvious function but he who considers the arterial wall as a cast iron pipe or an inert filter will make no lasting contribution to our knowledge of biology An increasing body of evidence points to great metabolic activity by the

arterial wall It burns sugar consumes oxygen and liberates carbon dioxide Furthermore it synthesizes cholesterol, phospholipids tri glycerides mucopolysaccharides proteins and many other substances It is capable of various degrees of regeneration and it responds promptly to various types of stimuli In short it has many specialized functions and all the attributes of an organ

TABLE I

ESTIMATE OF PERCENTAGE COMPOSITION OF TISSUE IN VARIOUS TYPES OF VESSELS

	Elastic arteries (%)	Muscular arteries (%)	Arterioles (%)	Capillaries (%)
Endothelium	2	5	5	40
Reticulum	3	5	5	40
Collagen	5	10	10	10
Elastin	*20*	*5*	*5*	—
Smooth muscle	20	40	40	—
Areolar tissue	20	5	5	—
Ground & cement substances	30	30	30	10

TABLE II

ESTIMATE TOTAL SIZE BY COMPONENT TISSUES IN GRAMS

	Endo thelium	Reticu lum	Col lagen	Cement & ground sub stances	Elas tin	Smooth muscle	Areo lar tissue	Total
Elastic arteries	2	3	5	30	20	20	20	100
Muscular arteries	15	15	30	90	15	120	15	300
Arterioles	50	50	100	300	50	400	50	1000
Capillaries	1200	1200	300	300	—	—	—	3000
Total	1267	1268	435	720	85	540	85	4400

I will not go into all the histologic and ultramicroscopic details of this organ but suffice it to state that they yield confirmation for meta bolic activity in the form of mitochondria Golgi apparatus and endo plasmic reticulum

The arterial wall is the only pulsatile organ in the body and the full significance of this unique property has not been appreciated If we look at a cross section of a portion of this organ we can imagine *not only* a primary pulse from the lumen but a counterpulse from the vasa vasorum and I cannot help but believe that this turbulent environ

ment affords opportunities for physico chemical activity that are lacking or limited in other parts of the body

Time does not permit other considerations about this interesting organ In the remaining time allotted to me I would like to point out how some of the perplexities of atherosclerosis begin to resolve them selves when the arterial wall is looked upon as an organ in its own right

Local accumulation of lipid and other substances in other words the lesions of atherosclerosis can result from (*1*) Increased filtration (*2*) Increased local formation (*3*) Decreased local removal (*4*) Any disparity between these 3 factors that favors local accumulation

I have already presented data showing that passive filtration cannot be the total answer to the problems of atherogenesis This morning Milch Gould and others showed that simple building stones can be converted to cholesterol and other lipids under a variety of conditions I am sure that Drs Werthessen and Zilversmit who follow me will present evidence for biosynthesis of various lipids by the arterial wall The enzymic data of Gordon and others indicate metabolic work by the arterial wall and the electron micrograph reveals the presence of meta bolic machinery in the form of mitochondria Golgi apparatus and endoplasmic reticulum

The natural history of atherosclerosis as we see it at postmortem examinations in man further emphasizes that atherosclerosis is an active metabolic process under hormonal and other controls To date we have examined over 2000 aortas and several hundred coronary arteries from individuals ages 1–40 performed in 8 widely scattered geographic areas (New Orleans Spain England South Africa Guatemala Colombia Costa Rica and Puerto Rico) Our studies to date (4 5) have in dicated that atherosclerosis develops by definite sequential stages (1) fatty streak (2) fibrous plaques (3) complication (e g thrombosis or hemorrhage) and (4) clinical disease and that each stage is a neces sary precursor to the following one The factors responsible for suc ceeding stages may be and probably are different from those that initiated the first stage (atherogenesis) Thus intelligent therapy must take cognizance of these different stages because that which may be effective against one stage may be ineffective or even contraindicated against another stage

Most of our studies to date have been directed toward the first stage fatty streak as we are convinced that prevention or retardation of this stage will prevent or retard all subsequent stages *No fatty streak no fibrous plaque no fibrous plaque no complications no complications no clinical disease* If each link in this chain of pathogenesis is sound *no fatty streak no clinical disease*

Several findings of interest have emerged from these studies (3) in this age group up to 41 years

(a) Every child beyond the age of 3 years in all parts of the world thus far sampled despite the widely varying genetic and environmental backgrounds represented by the 8 geographic areas studied has shown some degree of sudanophilic fatty streaking of the aorta and this has been confirmed by histologic and histochemical studies This has raised *serious fundamental questions about the definition of normal" and* the relationship of diet to atherogenesis

(b) The degree of fatty streaking of the aorta in cases of sudden accidental death (automobile accidents gunshot wounds etc) has been as great or greater than it has been in cases of death due to natural causes Thus the terminal illness is not the determining factor

(c) In all parts of the world that have been studied thus far a rapid increase in fatty streaks (expressed as percentage of the inner surface of the aorta that stains red with Sudan IV) has occurred between the ages of 8 and 18 years This has suggested a relationship of athero genesis to the hormonal changes of puberty

Other studies related to race age sex principal cause of death and associated findings such as hypertension hypercholesterolemia diabetes weight gain or weight loss are in progress and will be reported as suf ficient data for statistical analyses become available The findings to date however are sufficient to warrant certain tentative conclusions

The tempo of the natural history of atherosclerosis is in terms of months years and decades—not that of 3 meals per day Our anatomic studies are more easily interpreted in the light of increased local forma tion of lipids (by mesenchymal cells in the inner layer of the arterial wall) and/or decreased local removal of lipids than they are in the light of filtration of lipids from the blood The evidence from our studies substantiates that from other sources in indicating that the arterial wall normally forms and turns over to the body economy (metabolic pool and fat depots) certain lipids not unlike those that are normally found in the blood and the local accumulation of these *lipids in the* arterial wall results from some breakdown in this normal turnover mechanism

If diet plays a role in atherogenesis and there is ample evidence that it does it does so not by flooding the filtering capacity of the ar terial wall with cholesterol chylomicrons or lipoprotein complexes but by *upsetting some of the safety valves (hormones? enzymes?)* that normally protect the arterial wall

In summary I would like to emphasize the role of the arterial wall as an organ and express the belief that progress in the conquest of

atherosclerosis will vary directly with our knowledge of the factors (hormonal and enzymic) that govern the metabolism of the mesenchymal cells in the inner layers of this pulsatile organ

REFERENCES

1 Duff G L and McMillan G C *J Exptl Med* **89** 611 (1949)
2 Holman R L Unpublished observations
3 Holman R L McGill H C Jr Strong J P and Geer J C *J Oil Chemists Soc* **34** 583 (1957)
4 Holman R L McGill H C Jr Strong J P and Geer J C *Lab Invest* **7** 42 (1958)
5 Holman R L McGill H C Jr Strong J P and Geer J C *Am J Pathol* **34** 209 (1958)
6 Kellner A Correll J W and Ladd A T *J Exptl Med* **93** 373 (1951)
7 McGill H C Jr and Holman R L *Proc Soc Exptl Biol Med* **72** 72 (1949)
8 Seifter J *Am J Med Sci* **232** 605 (1956)

CHAPTER II

Control of Aortal Lipid Metabolism and Lipid Movement by Hormones and Vitamins

N T WERTHESSEN

Southwest Foundation for Research and Education San Antonio Texas

To facilitate matters for the discussion that may ensue at the end of this presentation and to lead into the presentation of the work in our laboratories that is germane to the subject matter of this conference two points of background information must be presented

The first item is the technique employed We perfuse aortas However we do not perfuse them as a perfusion is commonly understood We approximate culturing the organ to the extent that growth and repair of structure are seen grossly Dr Holmans group is primarily interested in organ and cellular architecture Consequently for our effective collaboration it was a matter of some moment to them whether or not the aorta we maintained *in vitro* bore any resemblance to the aorta as they knew it *in situ*

When a calf's aorta is mounted on the pump the vasa vasorum are seen as fine red lines on the surface of the vessel The arteries are ligated at a sufficient distance from their root in the aorta to avoid injury to the origin of the vasa That the vasa are patent is checked by first flushing them with Tyrode solution and then observing their filling with blood when the perfusion begins

Studies by Dr Holmans group of perfused calf aortas have shown that there are as would be expected areas of damage in the cells It is also clear from these same studies that there is a great deal of healthy living tissue (2) Electron microscope studies now in progress are confirming the earlier work

This brings us to the second point to be made That is that the procedure of taking an organ out of the body stripping it of all connective tissue pushing and pulling on it in the fashion requisite to get it clean tie up the efferent vessels and finally to get it into position in its glass container is inevitably a procedure that will traumatize the organ However the trauma is not so great as that which would occur if microorganisms were growing in the preparation in addition Then one does not need electron microscopy to show damage Finally the trauma in no way even approximates the trauma imposed on cells when they have been sliced into "tissue slices" kept ice cold for a while and finally suspended in Krebs buffer to do what the observer wishes to study

This factor of trauma is emphasized here because we feel that a neglect to recognize it has had a tendency to place emphasis on certain organs and their contribution to the body's economy as regards lipids that might not otherwise have occurred Also we wish to have it recognized that in our own work it is an injured organ which is responding to the agents and conditions to be described Consequently we may be emphasizing certain facets of aortal function which *in situ* are not as prominent

The studies to be discussed were initiated at the Worcester Foundation when Dr Schwenk and the author (1) were able to show that in this system of perfusion the heavily traumatized liver rather than

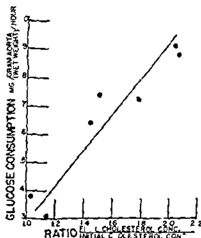


FIG 1 Glucose consumption Relation between consumption of glucos by the aorta and ratio of the final concentration of cholesterol found within the aorta to initial concentration

the more gently treated made radioactive cholesterol from C^{14} labeled acetate most efficiently This finding suggested reinvestigating the capacity of a number of other organs including blood and the aorta to convert labeled acetate into labeled cholesterol We found the capacity present in all those examined

Later studies in collaboration with Dr Milch (4) demonstrated that not only did the aorta synthesize cholesterol from acetate but it also accumulated cholesterol while being perfused Figure 1 shows the relationship found between glucose consumption by the aorta and the accumulation of cholesterol in 72 hour experiments The relationship suggests that cholesterol accumulation under these conditions is a dynamic process

At this point we decided to revise our approach We placed our

prime emphasis on the question "Can the lipid metabolism in the aorta be regulated in any way?"

Blood pressure as a possible control was first studied The average of the systolic and diastolic levels of the lower range of pressures was approximately 100 mm of Hg that of the higher was about 200 mm Each experiment was run for precisely 24 hours At zero time carboxyl labeled acetate was added to the system

The results are shown in Figs 2 and 3 and Table I From these it is apparent that (*a*) under hypertension the accumulation of cholesterol (ΔC) in the aorta is predictable and that this accumulation is a func

TABLE I

ACCUMULATION OF C^{14} FROM ACETATE INTO AORTAL CHOLESTEROL DURING 24 HOUR EXPERIMENTS UNDER HYPERTENSIVE AND NORMOTENSIVE CONDITIONS MEASURED BY DETERMINING TOTAL RADIOACTIVITY COUNT PER MINUTE OF THE CHOLESTEROL IN THE ORGAN

Perfusion no	Glucose consumption mg /gm wet weight/ 24 hours	Total count per minute of aortal cholesterol (TCa) × 10	Total count per minute of perfusate cholesterol (TCp) × 10	Total count per minute of cholesterol in system (TCs) × 10
High pressure				
216	12 8	186	25	211
221	13 1	190	181	371
224	20 2	70	15	85
226	16 6	108	38	146
228	25 9	71	15	86
229	38 3	166	74	240
240	23 6	84	14	95
245	22 2	151	89	240
248	23 3	105	111	216
250	34 1	311	43	354
254	19 4	143	27	170
Average high pressure	22 7	144	57	
Low pressure				
215	20 6	589	22	611
249	33 8	229	101	330
251	16 3	330	51	381
266	21 4	499	118	617
330	21 8	365	18	383
336	31 9	152	265	417
332	20 9	285		
Average low pressure	23 8	350		

tion of the initial concentration (C_1) (*b*) at 100 mm of Hg there is no such predictability (*c*) from the significant difference in C^{14} titer of the cholesterol obtained from these aortas there is additional proof

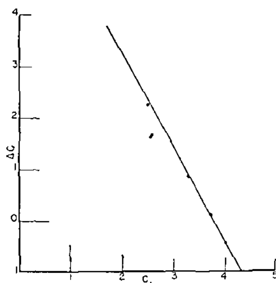


Fig 2 *Relationship at high perfusion pressure (200 mm Hg)* of the change in concentration ΔC to the original concentration C_1 of cholesterol in the aortic wall expressed in mg/gm dry weight Each point represents one perfusion which lasted precisely 24 hours

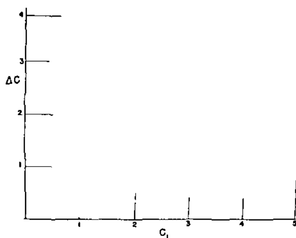


Fig 3 Relationship at low perfusion pressure (100 mm Hg) of the change in concentration ΔC to the original concentration C_1 of cholesterol in the aortic wall expressed in mg/gm dry weight Each point represents one perfusion which lasted precisely 24 hours

from an entirely different parameter that pressure alone is able to influence markedly the metabolism of cholesterol in the aorta

From other experiments we had found that estrone could over a 72 hour period of administration to a perfusion alter the aorta's incorporation of C^{14} into cholesterol These data plus the results of other workers suggested extending the observations to cover more lipids than cholesterol alone (5) Dr Nyman of our group thereupon devised a

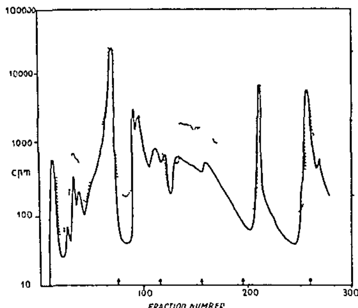


Fig 4 Duplicate chromatograms of the same sample of aorta lipids Each curve represents the chromatographic analysis of the lipids from 1 g of aorta tissue It will be noted that the ordinate (cpm) is plotted on a 4 cycle logarithmic scale which minimizes the dramatic change in count from fraction to fraction

In addition to showing the reproducibility—the dotted curve has been shifted upwards slightly to facilitate comparisons—the two graphs illustrate the effect of the rate of solvent flow on the separation attained The first half of the chromatogram represented by the solid line was run more slowly the last half more quickly than the chromatogram shown as a succession of dots It will be seen that the sharpness of the peaks is increased as the solvent flow is decreased

chromatographic procedure using a silica gel column and various eluates to separate out a number of lipid fractions He relied on a Tri Carb scintillator to measure the C^{14} in the eluates taken from the column These totaled about 300 and produced what we can call a lipid profile of radioactivity (3) This profile is shown in Fig 4

The profile indicates that the number of lipids as chemical entities

that are synthesized by the aorta is very large Yet later experiments showed that this profile contains only about one half of the total radioactivity that can be found in the total lipid fraction of an aorta when that fraction is defined as material soluble in Delsals solution This profile is of interest from the point of view of the number of lipids synthesized *However it was too complex and laborious to employ* in a routine assay procedure

Accordingly the chromatographic technique was modified to permit simplification and rigid standardization A system that produced 4 peaks of radioactivity was selected To set up a bioassay for chemical agents we investigated on both a qualitative and quantitative basis the relationship between the upper and lower halves of a calfs aorta as to their manner and ability to incorporate C^{14} from labeled acetate into lipids Qualitatively they do the same thing Quantitatively they do *not* as will be shown later But we were able to design an assay technique

Both portions are now mounted simultaneously in 2 separate perfusions Both portions are nurtured by 500 cc aliquots of a perfusate that has been made up as a single batch Consequently from the bioassay point of view we can state that we are using the same blood and the same aorta as both control and experimental subject The synthesis that goes on in the blood should obviously be the same in the 2 perfusions However the top and the bottom of the aorta do not contain the same amounts of connective tissue per gram Consequently it was necessary to obtain a description of their interrelationship

This description has been constructed on the simplest of terms The aortal halves as removed from the pump are weighed Each is then extracted for its lipids and the extract treated to remove unused labeled acetate An aliquot of this total extract is taken and reserved for counting The extract is called TL_D indicating that it is the totality of lipids obtained from the aorta and defined as those obtained by the Delsals technique of extraction

A second aliquot of the TL_D is placed on the chromatographic column and treated according to the prescribed technique The eluates are counted and the amount of count in each of the 4 peaks determined The sum of the count in the 4 peaks or the total count that comes off the column is called TL_C indicating that it is the total lipid as defined by chromatography TL_C as stated above may contain one half the C^{14} titer of TL_D

The two values TL_D and TL_C are then divided by the weight of the portions from which they were obtained and we then have the values TL_D/gram and TL_C/gram for the top and bottom halves of the aorta

Figures 5 and 6 show the original 10 experiments that established the relationship and the lines for 2 and 3 times the standard deviation of the computed line Repeat experiments 18 months later fitted even better than the original points

Given these curves it was possible to determine by adding an agent to one half of an aorta whether its lipid metabolism on a per gram basis defined as either TL_D or TL_C was affected by the agent In addition it was possible to determine the normal distribution of the 4 peaks in TL_C These were found to be decently stable as indicated in Table II

TABLE II

THE RATIO OF THE C^{14} COUNT FOUND IN THE 4 PEAKS OF A CHROMATOGRAM OF THE AORTAL LIPIDS TO THE TOTAL COUNT IN THE 4 PEAKS OR TL_C
(The Values and Their Standard Deviations Were Obtained by Analyses of 19 Control Portions of Aortae)

Peak	Ratio and standard deviation
I	$0\,47 \pm 0\,070$
II	$0\,40 \pm 0\,061$
III	$0\,04 \pm 0\,013$
IV	$0\,09 \pm 0\,027$

To present the data obtained in this study we have made use of the fact that the control in this particular assay predicts what the experimental portion should have shown Knowing what it should have shown permits a comparison between the expected" and the "found" value Thus in Table III where the effect of various agents on the aorta in this system is presented the values given are those showing the degree of departure from the expected value No effect is reported in the table unless it was found to be significant in its deviation in 2 experiments That is in each of the 2 experiments the single deviating value had to be so far from normal that it lay in a range where the p value for it to be normal was less than 0 05

This criterion of significance is very rigorous It tends to remove from the table a considerable number of differences that could be analyzed for significance if the average value of the controls was compared with the average value of the assays in a more standard analysis such as the *t* test

The technique of assay permits examination of another facet of the over all metabolism of lipids in the aorta that is not too often considered—namely movement of lipids *out* of the aorta There is a great deal of discussion that one can read as to how they move *in* It is our sentiment that the manner of movement of lipids in and out of the aorta is a subject that should be exhaustively studied

Studies such as those reported by Dr Gould and Dr Milch and others show that labeled lipids introduced into the circulation are to be found in the wall of the blood vessel Perhaps they did filter into the region We prefer for the moment at least to assume that the process is one which is a bit more complex than the word filtration" connotes

To make this point a bit more strongly let us first remind you of our first finding that the accumulation of cholesterol by the aorta was

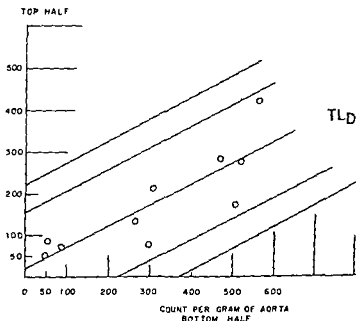


FIG 5 The relationship between the upper or cardiac half of the aorta and the bottom or abdominal portion of the aorta when compared on the basis of the count in the TL_D extract per gram of aorta The solid lines encompassing the points give the 0 05 and 0 01 probability levels respectively The equation of this relationship is $y = 0.52x + 19$ where $y = TL_D$/gram of upper half and $x = TL_D$/gram of bottom half

directly related to the amount of glucose the aorta consumed If that cholesterol was filtered in from the medium then the aorta was apparently engaging in more work as more filtered into it Secondly an experiment employing various concentrations of serum in Whites solution is here very much to the point

In Fig 7 is plotted the ratio (R) of the C^{14} content of cholesterol in the perfusate as related to that in the aorta (open circles) and to the whole system (solid circles) In these experiments since there were no formed elements of the blood in the perfusate the perfusate could

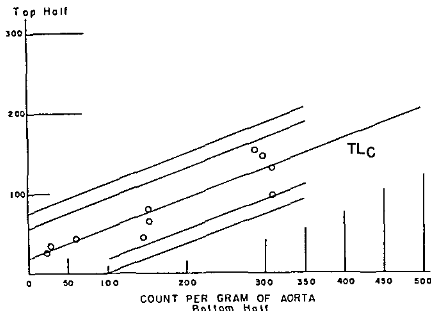


FIG 6 The relationship between the halves of the aorta as shown in Fig 5 except that here the extract used in obtaining TL_D has been chromatographed to yield TL_C (see text) The 0 05 and 0 01 lines of probability are shown The equation of the relationship is $y = 0.38x + 18$ where $y = TL_C$/gram of the top half and $x = TL_C$/gram of the bottom

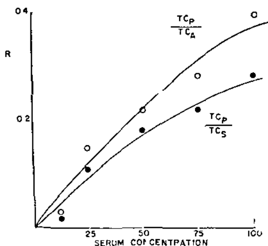


FIG 7 The value of the ratio R relating the total count of cholesterol in the aorta (TC) and the total count of cholesterol in the perfusate (TC_p) as TC_p/TC when examined as a function of the serum dilution employed in the perfusate R is also shown for TC_p/TC where TC is the total count of the system i e TC + TC_p

TABLE III

THE EFFECT OF CHEMICAL AGENTS ON THE VARIOUS PARAMETERS OF LIPID METABOLISM DESCRIBED IN THE TEXT

Mg of agent tested per 500 ml of perfusate per experiment		Aortal parameters			Perfusate parameters	
		TL_D	TL_C	Peak ratios	TL_C	Peak ratios
Testosterone	30–1 7	2E[a]	2E[a]	—	—	—
Seifter and Baeder s lipotrophin	5 6–5 6	—	2E[a]	—	—	—
Hydrocortisone	10–10	—	2E[a]	Peak 2 at 0 6E[a]	—	—
Estrone	9 2–0 7–0 5	—	—	Peak 4 at 2E[a]	—	—
Progesterone	10–10	—	—	Peak 4 at 2E[a]	—	—
p Aminobenzoic acid	102–111	—	—	Peak 2 at 0 6E[a]	2E[a]	Peak 4 at 9E[a]
Nicotinic acid	100–100	—	—	—	0 5E[a]	Peak 2 at 0 6E[a] Peak 3 at 0 6E[a]

[a] The letter E in the table refers to the result predicted by the control Multiples or fractions of E demonstrate that the observed result deviated from the expected value for that particular parameter to the extent shown and that the effect was observed to be statistically valid in each of the experiments Where no effect is recorded in the table no change was seen or observed only once

not incorporate C^{14} into its cholesterol Yet we see that as the percentage of serum in the perfusate rose so did the amount of C^{14} labeled cholesterol in the perfusate Or in other words as the percentage of serum rose so did the movement of newly synthesized cholesterol out of the aorta and into the perfusate

Coming back now to the assay technique The fact that perfusate from the same batch was used to perfuse each half of the aorta permitted us to compare the lipid moieties of the perfusate to determine whether movement from the aorta had occurred Two fairly obvious sources of error must be borne in mind when making the comparison The first is the chance that the agent could induce the aorta to synergize synthesis in the perfusate the second that it could act on the perfusate alone The second item is easily checked The first is not but can for the present be considered as having a low probability of existing

Table III summarizes almost completely our results studying various hormones and vitamins The two vitamins have now been shown in one experiment each to be active at the 5-mg level Niacin showed lesser effect PABA (para aminobenzoic acid) was still very potent at that dosage level

In evaluating the data it should be recalled that the criteria of an effect are extremely rigorous and that buried in these data are probably a great number of interesting capacities

However the objective of the study was to determine if substances known to be able to control biochemical systems (such as vitamins and hormones) could affect lipid metabolism in the aorta and movement of lipids between it and its nutrient fluid The data that are presented would seem to be sufficient to settle that point

REFERENCES

1 Schwenk E and Werthessen N T *Am J Physiol* **171** 55 (1952)
2 Strong J P McGill H C Jr and Nyman M A *Circulation Research* **5** 294 (1957)
3 Werthessen N T Hahn J W and Nyman M A *Intern Conf Biochem Problems Lipids* (1956)
4 Werthessen N T Milch L J Redmond R F Smith L L and Smith E C *Am J Physiol* **178** 23 (1954)
5 Werthessen N T Nyman M A Holman R L and Strong, J P *Circulation Research* **4** 586 (1956)

DISCUSSION OF PAPERS BY DRS HOLMAN AND WERTHESSEN

POPJÁK What was the perfusate? Was it blood or some other fluid?

WERTHESSEN The perfusate consists of defibrinated blood diluted 50% with Wright s solution It is used betwen 64 and 72 hours after drawing from the donor

FREEDBERG Does the fluid contain white cells or platelets?

WERTHESSEN The white cells and platelets are probably very much reduced in quantity because of the defibrination

POPJÁK In view of the fact that James and Lovelock have demonstrated that red cells *in vitro* can synthesize a considerable range of lipids which are very *rapidly transferred to plasma lipoproteins* is it conceivable that the results you are getting in the perfused aorta might be explained by the transfer of lipids synthesized in the red cells by lipoproteins in the blood serum?

WERTHESSEN No this is not possible We have a number of experiments using serum alone as perfusate and cholesterol and lipids were synthesized by the aorta It is true that the amount of synthesis that goes on in the red cell is perfectly fantastic One of the interesting points is that cholesterol esters are not to be found Free cholesterol is found Very recent data indicate that it is only the free cholesterol which comes out of the aorta into the perfusate

KATZ Have you ever thought of using a small amount of cyanide in the perfusate of either the upper or lower aorta and noting what happens?

WERTHESSEN No we haven t We have checked this out in other ways This is background information that we have Dr Katz I can give it to you later

ADLERSBERG I have a question for Dr Holman In the age group data that you showed toward the end was there a separation by sexes—males versus females?

HOLMAN Yes we have those data We have the most data in the New Orleans area and the male and female negro behave alike The male and female *whites are similar The greatest discrepancy is between negro female and white female* the white female being the least involved of the four groups and the negro female not showing quite the drop that the negro male showed

RALL What fraction of the labeled acetate Dr Werthessen was incorporated into the total lipid say in a 24 hour period?

WERTHESSEN It is of the order of 1%

OLIVER *I think a lot of people here will agree with these four stages which* Dr Holman elaborates A small criticism might be that he relates these stages to precise age groups He will have us believe that there is formation of fat streaks from age 0 to 10 that this becomes irreversible at about 20 fibrotic at 30 and by the time we reach 40 or more the clinical complications set in While this sequence is undoubtedly true all this takes a long time and a collateral circulation has time to develop Now there are a number of situations where hypercholesterolemia will develop comparatively acutely These are largely endocrine disturbances such as following bilateral oophorectomy diabetes and myxedema—all conditions where we know there is an increased incidence of clinical coronary disease and of coronary atheroma Thus in middle age the onset of hypercholesterolemia and perhaps of fatty streaks may occur over a shorter period than these stages indicate Might it not be the case that we should think in terms of reversibility of fat deposition on the arterial wall at a later age than you suggest? We must not just assume that at middle age we are dealing with a fibrous lesion and that all the work of developing methods to lower cholesterol levels is in vain One cannot but be impressed by some of the observations that have come out of the prisoner of war camps in Europe when in association with extreme starvation there was remarkable freedom from coronory and aortic atheroma

HOLMAN I agree with your statement about diet It is obviously an over simplification of the problem to divide the lesions into four stages One stage does not suddenly stop and another begin The formation of fatty streaks does not end at age 20 25 or 30 Some cases even in advanced ages show fatty streaks

in the process of development Now to come back to your second question implying that hypercholesterolemia alters the natural history of this sequence of events the evidence for this implication must be reckoned as presumptive rather than proven When we see fatty streaks developing in all individuals above 3 years of age (and frequently before age 3) it is difficult for us to believe that each of these children has had hypercholesterolemia hypertension or some major upset in lipid metabolism It is much easier for us to think of this process as being "normal and of the arterial wall as being a factory in its own right In other words the arterial wall normally forms and turns over to the body economy (metabolic pool and fat depots) lipids not unlike those normally found in the blood

VOICE I would like first of all to express my pleasure at seeing a reminder of the stages of atherosclerosis and their evolution from fatty streaks to pearly plaques containing lipid in the base to superimposition to further lipid vascularization and development of complications in clinical disease I think it is a healthy point in this conference for us to be reminded of this and to be reminded of the fact that an ability to stop or reverse the process at any of the earlier stages is all important for the check of the later stages Now with regard to that I think Dr Holman s data particularly on the international comparison point up and focus on the area of concentration and I would appreciate his comment on this namely what is responsible for the regression of streaks and the failure of pearly plaques to develop when that happens and on the other hand what is responsible for the further development of plaques and the superimposition of further lipid on those plaques? I think that is a critical question Those of us who have worked on atherosclerosis and have said that this is the ground lesion the fundamental for the whole process welcome this and point out that these are very similar to what is studied in experimental animals It is of great interest to us with respect to the findings in the negroes versus the whites in New Orleans and particularly the findings on the negro women that these findings anatomically parallel the vital statistics in a city like Chicago and in the United States as a whole namely a not dissimilar finding with respect to death rates in middle age in the 40 s and 50 s in negro and white males due to coronary disease and a much higher rate of clinical coronary disease in negro women as a cause of death than in white women One can think of at least two possible explanations as hypotheses in this regard although there are certainly other factors to be considered namely the much greater incidence of hypertension in negro women and the much greater incidence of obesity in negro women Now with respect to this set of data I wonder if Dr Holman could expand on the situation in the coronaries as paralleled or not paralleled in the aorta

HOLMAN That is quite a big order Your first question is the $64 000 question and your second question is the $164 000 question These are the two questions for which we would like to have answers The critical point of interest in our research study at the present time pertains to the two fates of the fatty streak (1) progression to fibrous plaque or (2) reversibility and disappearance I have been trying to entice some of my biochemical friends into doing microchemical analyses of the lipids at these two ends of the fatty streak If the chemical composition of these two ends is the same our problem is one thing if it is different our problem is something else We are stymied for lack of these chemical analyses but I am still hopeful that we will be able to get them

Your second question is more to the point and is subject to partial answer We have only recently started expanding our studies to the decades beyond 40 years

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POPJÁK In view of the fact that James and Lovelock have demonstrated that red cells *in vitro* can synthesize a considerable range of lipids which are very rapidly transferred to plasma lipoproteins is it conceivable that the results you are getting in the perfused aorta might be explained by the transfer of lipids synthesized in the red cells by lipoproteins in the blood serum?

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CHAPTER 12

Phospholipid Turnover in Atheromatous Lesions[1]

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In the study of atherogenesis the cholesterol fed rabbit has played a significant part This animal exhibits early and pronounced hyper cholesteremia and hyperlipemia which is followed by clearly visible accumulations of sudanophilic materials in the aortic arch and in the descending aorta The fact that this disease is produced by cholesterol feeding and that, in the early days cholesterol was more easily detect able than any of the other lipid fractions has given the impression that atherogenesis is initiated and maintained by an abnormality in the metabolism of dietary or endogenous cholesterol It seemed quite nat ural to assume that, in the rabbit at least, the dietary cholesterol ac cumulates in blood and is then deposited in the arterial intima However the sharply circumscribed localization of these lesions is not easily reconciled with the idea that fat deposition is a result of "col loidal instability" of blood lipids since on the basis of this theory one would expect a fairly uniform accumulation of arterial lipids An ab normality in the metabolism of arterial tissue might on the other hand reveal the reasons for a local aggregation of lipids The fact that several investigators have found considerable amounts of phosphatides in ar terial lesions stimulated us to study the factors influencing the accumula tion of phosphatides in the arteries of cholesterol fed rabbits

Usually atherosclerotic rabbits were produced by the addition of 1 g of cholesterol dissolved in 2 8 g of hydrogenated fat to the daily ration of each experimental animal for a 3-5 month period Suitably matched controls were maintained for the same length of time on Purina rabbit chow It was shown that fat alone does not raise blood lipids or produce arterial lesions In contrast, dietary cholesterol apparently not only raises the level of blood cholesterol but also markedly increases the plasma phosphatide concentration (Table 1)

This in itself is a somewhat curious phenomenon since it is not clear why cholesterol feeding should raise the level of lipids other than cho lesterol The interaction of serum lipids in normal and pathological

[1] These studies were supported in part by grants from the Life Insurance Medi cal Research Fund and from the Heart Institute of the National Institutes of Health (H _181)

Cholesterol for these studies was donated by Merck and Company

and to include estimates on the coronary arteries The problem of quantitating the lesions becomes more complicated as the more advanced lesions are studied Despite this I believe our data are sufficient to state that in the New Orleans area roughly 1 out of 5 of the fatty streaks has become a fibrous plaque by age 40 and that the fibrous plaques rarely develop from fatty streaks in less than 15 to 20 years The changes in the coronary arteries follow those in the aorta by about a decade and are positively correlated with those in the aorta in all 5 decades from 30 to 80 in the New Orleans area There are exceptions to this general statement and we do not have enough data to draw line graphs for the coronary artery lesions such as those that I have shown you for the lesions in the aorta

If we shift from the New Orleans area down to Guatemala we fail to get this positive correlation between coronary and aortic atherosclerosis Thus the failure of the Guatemalan Ladinos to develop myocardial infarction may be related to their failure to convert fatty streaks to fibrous plaques This illustrates again that factors pertaining to blood coagulation may be more important in precipitating clinical disease than are factors pertaining to atherogenesis—but the fact still remains that if there had been no fatty streak there would have been no fibrous plaque and the factors pertaining to blood coagulation would not have been operative

FREEDBERG Dr Oliver has apparently made the point that some alteration in hormones does produce atherosclerosis Now he mentioned that myxedema produces atherosclerosis We have looked the literature over very carefully on this subject and I believe that there isn t any substantial evidence that would favor the concept that hypothyroidism in man will produce atherosclerosis Does any body know of any evidence showing that myxedema alone will produce atherosclerosis in experimental animals? Then the second point I wanted to make for Dr Holman is that there was a paper published by Dr Gottlieb some 2 years ago showing an increased incidence with increasing age of what he described as arteriosclerotic plaques in the aorta of the pig My recollection is that these are not fatty but are fibrous Is that correct?

HOLMAN I have gone over these lesions with Dr Gottlieb and they are practically all fibrous intimal thickenings or areas of medial necrosis with varying degrees of calcification and practically no lipid As far as I know no disease is ever related to these lesions

states is poorly understood It would seem reasonable to assume that whenever the blood and liver are presented with an excessive amount of one lipoprotein constituent such as cholesterol they must either excrete or deposit this cholesterol or transform it by oxidation or by binding to a phospholipid protein complex (Fig 1)

In the latter instance one would expect an increase in serum phosphatides along with an increase in cholesterol concentration The choice of these pathways may be dictated by the status of the internal environment which in turn probably depends upon both genetic and environmental factors One species like the dog may have solved the excess

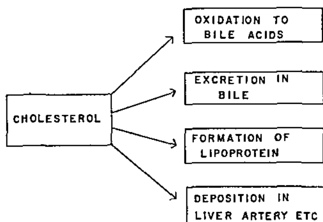


FIG 1 Schematic presentation of possible routes of disposal of dietary and endogenous cholesterol At our present state of knowledge it is not possible to relate the four routes and they are therefore shown as independent processes

cholesterol problem by the formation of oxidative products such as bile acids whereas the rabbit stumbled upon the formation of excess lipoproteins and the deposition of cholesterol in liver and arteries That endocrine organs play a decisive role in the partition of cholesterol between these paths is well illustrated by the fact that suppressing the thyroid of the dog (7) or depriving the animal of glucocorticoids (3) seems to shift the metabolic picture in the direction of that of the rabbit Similarly the alteration of gonadal secretions is known to alter both serum lipid concentrations and the tendency toward atherogenesis (1)

Referring once more to Fig 1 it could be noted that an organism like the rabbit might "dispose of cholesterol by complexing it with lipoprotein and by depositing cholesterol in some of the soft tissues These two processes may be entirely independent and it is not necessary to look upon the increase in serum lipoproteins as the cause of

TABLE I

PLASMA AORTA AND LIVER PHOSPHATIDE CONCENTRATIONS AT VARIOUS INTERVALS AFTER BEGINNING OF AN ATHEROGENIC DIET[a]

Months on diet	Plasma Control	Plasma Cholesterol fed	Aorta Control	Aorta Cholesterol fed	Liver Control	Liver Cholesterol fed
1	3 88 ± 0 42	28 1 ± 7 1	16 0 ± 4 33	18 9 ± 5 9	101 ± 9 2	89 ± 9 2
2	4 42 ± 0 74	25 2 ± 3 3	17 7 ± 1 3	37 2 ± 7 9	100 ± 11 8	107 ± 4 0
3	4 14 ± 0 68	34 9 ± 3 8	17 2 ± 2 2	66 3 ± 12 1	130 ± 9 0	133 ± 6 0
5	4 68 ± 0 42	33 0 ± 3 1	14 7 ± 4 1	52 0 ± 4 6	112 ± 3 3	100 ± 9 9

[a] All values are expressed as mg phosphatide P per 100 ml plasma or 100 g of fresh tissue Mean values and standard errors are derived from groups of three animals

ment was compared to that derived from the injection of P^{3} phosphate which measures primarily arterial phosphatide synthesis Figure 2 indicates that nearly 90% of the arterial phosphatide P^{3} in the latter experiment was derived from synthesis *in situ* (6)

In a similar series of animals injected with P^{3} phosphate the specific activities of individual aortic phosphatides were compared to that of corresponding plasma phosphatides at an early interval after isotope injection (4) In nearly each instance the isotopic concentration of phosphatide (specific activity) in the arterial wall exceeded that of the plasma at the end of the experiment (Table II) Since the isotope concentrations at this early interval in both plasma and aorta were

TABLE II

SPECIFIC ACTIVITIES[a] OF AORTIC AND PLASMA PHOSPHATIDES 6 HOURS AFTER P^{3} ADMINISTRATION TO CHOLESTEROL FED RABBITS (4)

Cephalin		Lecithin		Sphingomyelin	
Aorta	Plasma	Aorta	Plasma	Aorta	Plasma
7.33	5.64	4.94	4.50	0.82	0.59
8.66	3.72	6.38	2.27	1.45	0.38
5.82	4.23	3.39	2.38	0.46	0.31
7.14	4.61	6.38	2.44	1.16	0.47
8.18	5.14	5.23	3.04	0.30	0.42
9.82	5.80	5.89	3.07	0.89	1.20

[a] Specific activities are given as per cent of the injected P^{3} per g of phosphatide P

still increasing with time this again showed that aortic phosphatide P^{3} was not derived from preformed circulating phosphatides One might object that the observed differences in specific activity between aorta and plasma were not very great and that the uncertainties of chemical fractionation of tissue phosphatides might obscure underlying similarities in the isotopic composition of plasma and aorta To investigate this problem further we studied the phosphatide metabolism in cholesterol fed eviscerated rabbits Removal of liver intestines and kidneys of the rabbit lowers the synthesis of plasma phosphatides from P^{3} *phosphate to less than 10% of normal (8) It thus becomes* possible to study the incorporation of P^{3} into aortic phosphatides while the isotope concentrations in the plasma phosphatide fractions stayed at extremely low levels

Table III shows a comparison of total phosphatide specific activities in the plasma aortic intima and in the rest of the arterial wall in completely eviscerated and sham operated rabbits 4 hours after injection of P^{3} phosphate The specific activity of arterial phosphatides in the

arterial lipid deposition Indeed the work which we shall now discuss briefly appears to show that although aortic cholesterol may well be derived from the blood it seems unlikely that the atheromatous lesions are formed by the deposition of intact serum lipoprotein molecules This conclusion is drawn from evidence that in the cholesterol fed rabbit the aortic phosphatide is derived from synthesis in the aortic wall A glance at Table I shows that the feeding of 1 g of cholesterol daily *to an otherwise normal rabbit causes a great rise in serum phosphatides* This increase in serum phosphatides clearly *precedes* any noticeable

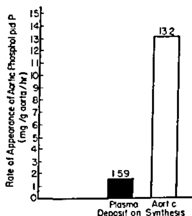


FIG 2 Relative rates of deposition and synthesis of aorta phosphatides Deposition of plasma phosphatides was calculated from the injection of P³ labeled plasma lipoproteins whereas an estimate of synthesis was obtained from the incorporation of P phosphate into aortic phosphatide after correction for deposition (6)

increase in aortic phosphatides What would seem more logical than to postulate that the aortic phosphatides were direct precipitates or filtration residues of the phosphatides in plasma? This conclusion is however not supported by metabolic studies with radioactive isotopes

Synthesis of arterial and of plasma phosphatides was measured by the incorporation of P³ phosphate into the various phosphatide fractions Although this technique may not under all circumstances give an adequate picture of phosphatide synthesis from fatty acid precursors the simplicity of P³ measurements and the relatively simple scheme of P intermediates as compared to those derived from C^{14} precursors makes this isotope quite suitable for the study of phosphatides in the aorta One of the first experiments to test the "deposition hypothesis" was performed by measuring the accumulation of the phosphatide P³ in the aorta of recipients infused with P³ lipoprotein This measure

We have previously speculated that indeed the increased aortic phosphatide synthesis might be a specific response to the accumulation of excessive cholesterol a response designed to remove this lipid by solubilization We have not yet succeeded in testing this hypothesis in a meaningful manner but one wonders why one should not then see a similar response in the cholesterol fatty liver In this organ of the *cholesterol fed rabbit neither concentration nor specific activity of* phosphatide differs from the control value (4) although liver cholesterol is greatly increased In the cholesterol fed rat, even some depression of liver phosphatide synthesis has been claimed (2, 5) Moreover the following evidence has been obtained to indicate that aortic phosphatide P^3 synthesis is not directly dependent on the serum lipid levels

Eight rabbits were divided into two groups One group was placed on a high cholesterol diet for 3 months and then on plain chow for 2 months while the other was maintained on plain chow for 2 months and on high cholesterol intake for 3 months Thus each group had received cholesterol for equal periods of time but at the time of sacrifice Group 1 exhibited almost normal serum lipid levels whereas Group 2 was severely hyperlipemic All animals received a dose of P^3 phosphate and were killed 6 hours thereafter for analysis of phosphatide P^{3*} in plasma liver and aorta Table IV shows the results of these analyses The serum cholesterol and serum phosphatide concentrations of the two groups differed markedly as did the liver lipid content The arteries of the two groups however appeared rather similar The cholesterol content of the whole thoracic aorta intima in the lipemic group was 31 5 mg and in the normolipemic animals 35 9 mg Apparently the 2 month period of cholesterol abstinence did not alter the cholesterol content or the gross appearance of the lesions Although the specific activity of intimal phosphatides in the normolipemic group appears to be less than that in the hyperlipemic animals the incorporation of P^3 into the phosphatides of the aortic thoracic intima was the same in both groups and elevated several times above that found in the artery of normal rabbits Thus it appears that the mechanisms in the aorta that are responsible for the accumulation of aortic phosphatides may be initiated by hyperlipemia in plasma but that, once set in motion they are not easily stopped by a return to normal serum lipid levels It is of course possible that the original atherogenic stimulus was not related to the elevated serum lipid level and that the real stimulus was still present in the normolipemic group Whatever the case may be it seems that phospholipogenesis of the artery is not readily altered by the degree of hypercholesteremia If we accept the hypothesis that phospholipogenesis is part of the arterial disease we must conclude that

eviscerated group exceeds that of the plasma more than 50-fold Thus it seems inconceivable than an appreciable amount of the aortic phosphatide P^{32} should come from plasma. Similarly the incorporation of P^{32} into arterial phosphatides of the eviscerated animals is no lower than that of their sham operated counterparts If plasma phosphatides served as a source for aortic phosphatides one should expect a decrease in the P^{32} phosphatide content of the arterial wall in the eviscerated animal. Evidence from each experiment then is incompatible with the postulate of phosphatide or lipoprotein deposition and supports the thesis that all aortic phosphatides are synthesized *in situ*

TABLE III

Plasma and Aortic Phosphatide of Cholesterol Fed Rabbits

	Eviscerated		Sham Operated	
	Concentration[a]	Specific activity[b]	Concentration	Specific activity
Plasma	0.20 ± 0 08[c]	0 066 ± 0 021	0 28 ± 0 03	0 58 ± 0 10
Intima	1 01 ± 0 01	4 27 ± 0 68	0 79 ± 0 13	4 58 ± 0 78
Media + adventitia	0 37 ± 0 01	5 23 ± 0 56	0 26 ± 0 07	3 55 ± 0.23

[a] Concentrations are expressed as mg lipid P per g of fresh tissue or per ml of plasma

[b] Specific activities are given as per cent of the injected P^{32} per g of phosphatide P

[c] Mean values and standard errors represent three animals

Although in the rabbit atheroma phosphatide is generated by arterial synthesis little or nothing is known about the cause or causes for the disturbed equilibrium that leads to phosphatide accumulation. Per whole aorta the synthesis of phosphatides is surely increased However the isotope data furnishing this conclusion must be interpreted with caution At the moment one cannot exclude the possibility that some inhibition of phosphatide removal or breakdown is responsible for the excess phosphatide in the atheromatous lesion At early intervals after the injection of P^{32} phosphate the specific activity of aortic phosphatides is a fairly good measure of the per cent renewal of the total aortic phosphatide pool In the atheromatous aorta the total amount of phosphatide is increased 5–6 fold and its specific activity is about the same as in the normal artery One may thus conclude that roughly 5–6 times as much phosphatide has been synthesized per unit of time per whole diseased thoracic aorta It might thus seem that the aorta for reasons unknown as yet accelerates its production of phosphatides whenever injury of some sort occurs

this part of the disease is not easily arrested by reduction of serum lipid levels This observation if confirmed by further studies in experimental animals and man should have therapeutic implications concerning the treatment of atherosclerosis by dietary or endocrine means

Summary

Evidence is presented that in the cholesterol fed rabbit the aorta accumulates lipid plaques containing appreciable amounts of phosphatides By injecting P^{32} phosphate it is shown that the excess aortic phosphatide is derived from synthesis by the artery Incorporation of P^{32} into arterial phosphatide does not diminish when liver spleen intestine and kidneys are removed although the plasma phosphatide P^{32} is reduced to one tenth the original amount When cholesterol is removed from the diet and hyperlipemia disappears the lesions persist and phosphatide synthesis per whole thoracic aorta remains as high as that observed in the hypercholesterolemic animals It appears that a deranged lipid metabolism of the artery once set in motion is not readily reversed by a decrease in serum lipid levels

References

1 Adlersberg D *Am J Med* **23** 769 (1957)
2 Clement G LeBreton É Pascaud M Tubiana M *Compt rend* **236** 412 (1953)
3 DiLuzio N R Shore M L and Zilversmit D B *Metabolism Clin and Exptl* **3** 424 (1954)
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Discussion

STAMLER I would like to make one comment on Dr Zilversmit s cautious statement toward the end of his presentation Eventually in the rabbit lesions regress upon cessation of cholesterol feeding That regression as Anitschkow showed many years ago is a rather slow process taking many months If phospholipogenesis is continuing at the 2 month stage as Dr Zilversmit s data show very neatly it is doing then it in no sense represents a metabolic process which perpetuates and further increases atherogenesis For while his rabbits at 2 months (I would gather) showed no evidence of regression he could anticipate that with a longer time on a normal diet regression would take place

Now to pose a question would Dr Zilversmit conclude from the data which very exquisitely demonstrated marked phospholipogenesis from P^{32} phosphate by the

TABLE IV
LIVER, PLASMA, AND AORTIC LIPIDS OF HYPERLIPEMIC AND NORMOLIPEMIC RABBITS

	Hyperlipemia			Normolipemia		
	Phosphatide			Phosphatide		
	Concentration[a]	Specific activity[b]	Cholesterol concentration	Concentration	Specific activity	Cholesterol concentration
Liver	1.32 ± 0.12	11.5 ± 2.1	58.5 ± 12.6	1.31 ± 0.12	6.90 ± 0.39	5.61 ± 1.2
Plasma	0.33 ± 0.06	1.54 ± 0.18	21.4 ± 2.5	0.050 ± 0.004	2.78 ± 0.17	1.23 ± 0.32
Intima	1.01 ± 0.15	7.29 ± 0.90	81.0 ± 13.3	0.86 ± 0.10	4.76 ± 0.48	59.6 ± 5.0
Media and adventitia	0.40 ± 0.03	5.17 ± 0.20	20.5 ± 2.1	0.26 ± 0.01	4.11 ± 0.56	11.4 ± 1.3

[a] Concentrations are expressed as mg. lipid P or cholesterol per g. of fresh tissue or per ml. of plasma.
[b] Specific activities are given as per cent of the injected 1^{3} per g. of phosphatide P.
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Now to pose a question would Dr Zilversmit conclude from the data which very exquisitely demonstrated marked phospholipogenesis from P^{32} phosphate by the

aorta that therefore most or all of the phospholipid in the atherosclerotic aorta of the cholesterol fed rabbit is produced by aortic tissue synthesis? Does he believe that such data refute the concept that the elevated aorta lipids are derived from the plasma? Does he feel it is possible to resolve this problem definitively by this method?

ZILVERSMIT I should like to say first that I am not at all certain that the increased phospholipogenesis is part of the atherogenic process It may be a defense mechanism in the artery to remove cholesterol by solubilization In the latter case one might well expect that the increased phospholipogenesis would continue until all the cholesterol had been removed With regard to the second part of your question I will admit that one cannot rigorously exclude the possibility that, although nearly all of the P^3 phosphatide is derived from synthesis some or all of the extra chemical phosphatide in the lesion is derived from the blood lipids I would say however that in the absence of good evidence for phosphatide de position and with strong evidence that phosphatide synthesis from P^3 is accelerated in the lesion the most probable explanation is that the excess phosphatide is derived from synthesis *in situ*

STAMLER The acute study in which lipoprotein containing P^3 labeled phospho lipids was injected indicated only a slight uptake of P^3 from this source by the atherosclerotic aorta However can one from this finding rule out the possibility that over a chronic period of time weeks or months such P^3 labeled lipoprotein might not be deposited in considerable amount? Would it not be possible still? The acute experiment demonstrated *only* that *in a few hours* there is not a sizable deposition Is it sound to extrapolate from this to what might happen over a considerably longer period of time particularly in view of your own data showing a lag between the diet induced rise in plasma phospholipids and increase in the aorta phospholipids? Could these data not be interpreted as indicating a process *of gradual increase in aorta phospholipids based at best in part on the inability* of the aorta to dispose of excessive amounts of phospholipids continually reaching it from the hyperlipemic plasma?

ZILVERSMIT Although I do not believe that you are correct I think that there is reason here for some caution It is true that from a 6 or 10 hour experiment one cannot draw accurate conclusions as to what might happen over a 2 week or a 2 month period Yet it seems to me that as far as the P^3 portion of the molecule is concerned we have evidence that a lot of phosphatide is synthesized The phosphatide synthesis is about 5 to 6 times as rapid as in the normal aorta The specific activity ratio of aortic to plasma phosphatides in some eviscerated rabbits was close to 100 This it would appear to me constitutes good evidence that deposition of phosphatide is minimal At the moment I cannot think of an experi ment that would prove beyond doubt that all the excess aortic phosphatide in the lesion is generated *in situ*

MILCH In the instance of the normal or nonatherosclerotic rabbit aorta would you say that there exists a readily reproducible balance between phosphatide P^3 specific activities in the serum and the aorta?

ZILVERSMIT I don t quite know what you mean by balance

MILCH For instance then do you find nonatherosclerotic aorta phosphatide P^3 specific activity equal to serum phosphatide P^3 specific activity?

ZILVERSMIT That depends on the time interval after P^3 administration At the 12 hour or 24 hour time interval after P^3 the specific activity of blood phospha

tide will be much higher than that in the aorta If you go to a 2 hour interval I expect that there will be much more difference in favor of the aorta

MILCH Would not such indicate then an apparent movement of phosphatide from serum to aorta?

ZILVERSMIT No I don t think that is what it means It is just a coincidence that after the 6-hour period the two specific activity curves cross

MILCH I gather then that in contrast to direct evidence of pronounced phospholipid synthesis in the atherosclerotic aorta you have made no definitive demonstration of such extensive synthesis in the normal aorta You cannot say in the undiseased instance whether equal and simultaneous synthesis occurs at both sites or whether transfer between compartments occurs

ZILVERSMIT Yes we have this evidence In the completely eviscerated nephrectomized normal rabbit, there is the same difference in specific activity between plasma and the aorta as in the cholesterol fed animal Thus again appreciable transfer of plasma phosphatide to the aorta of the normal animal does not appear likely

EDER I had the impression that in the human atherosclerotic aorta the cephalin is present in relatively lower concentration than in the plasma

ZILVERSMIT No that is incorrect I said they were reasonably similar It is true in the rabbit aorta also that particularly as the lesion progresses the lecithin and sphingomyelin increase much more than the cephalin. In fact the cephalin does not increase very much at all. In the human, of course that is perhaps not too surprising, since there is not much cephalin in human plasma either It is also true that when you study these separate fractions you find a much greater increase in the synthesis (or the incorporation of P^{32}) of sphingomyelin than of cephalin

GOULD Have you tried the effect of insults to arterial wall other than cholesterol feeding such as freezing or damaging by various chemical agents for their possible effect on phospholipid synthesis?

ZILVERSMIT We have not done this in the arterial wall but we are working on the technique developed by Boucek and Noble of implanting sponges under the skin and getting proliferation of connective tissue there

Isotopic Studies of Cholesterol Metabolism in Man[1]

LEON HELLMAN AND R S ROSENFELD

Sloan Kettering Institute for Cancer Research New York New York

Because of the importance of cholesterol in the pathogenesis of vascular disease and its role as a precursor of hormones and other steroids of physiological interest a systematic investigation of normal and disordered human sterol metabolism was undertaken using both kinetic and "sterol balance techniques Radioactive carbon (C^{14}) or radioactive hydrogen (H^3) labeled cholesterol or sodium acetate were used to study synthesis absorption and subsequent metabolism

THE BIOSYNTHESIS OF CHOLESTEROL FROM ACETATE

The specific activity curves of plasma free and ester cholesterol following the administration of acetate 2 C^{14} are shown in Fig 1 It can

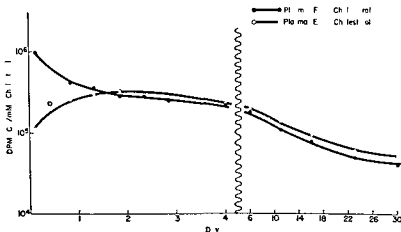


FIG 1 The specific activity curves of plasma free and ester cholesterol following the administration of acetate 2 C^{14}

be seen that the maximal value of the specific activity of free cholesterol is followed by a rapid decline The actual peak specific activity of free plasma cholesterol not shown in this chart occurs at 2 to 6 hours fol

[1] This investigation was carried out in part under contract AT(30 1) 910 with the United States Atomic Energy Commission and supported in part by research grants from the National Heart Institute and the National Cancer Institute of the National Institutes of Health of the United States Public Health Service

lowing oral acetate-2-C^{14}. The ester specific activity is at its minimal value with the earliest sample of blood taken at 3 to 8 hours. It then rises to a peak at about 2 days at which point it intersects the specific activity curve of the free cholesterol. After this point of intersection the ester cholesterol radioactivity declines at a similar rate to that of the free cholesterol and the specific activity of the ester cholesterol continues to exceed slightly that of the free cholesterol for the duration of the period of observation.

At 0.3 day after the administration of acetate, a point closely approximating the maximum value of the specific activity of free cholesterol, from 0.050 to 0.216% of the administered radioactive acetate had been incorporated per millimole of plasma free cholesterol. Corresponding values for plasma ester cholesterol at this point were lower, ranging from 0.017 to 0.068%. At the crossover point of free and ester cholesterol (about 2 days), where both fractions possess the same specific activity, from 0.032 to 0.118% of the administered dose was present per millimole of sterol.

Although the radioactivity of free and ester cholesterol declines at the same rate shortly after the intersection, the specific activities of these two forms of the sterol never again coincide in the normocholesterolemic subject. The finding that the specific activity of ester cholesterol is consistently higher than that of free cholesterol after the crossover point may be interpreted as suggesting that there is no equilibrium between the two fractions; in other words, ester cholesterol once formed from the free sterol does not reappear in the free cholesterol pool in normal subjects.

The decline of the specific activity curves shown in Fig. 1 can be resolved into a series of exponential rates (6). Until additional information is available concerning the magnitude of each of the many physiological processes that contribute to the composite series of rates observed in the specific activity curve, no definitive statement can be made about the turnover time or half-life of serum cholesterol studied in this manner. It might be assumed that the most rapid rate of $t_{1/2}^{c}$ = 0.20 to 0.47 day represents the conversion of acetate to cholesterol, while the intermediate rate of $t_{1/2}^{b}$ = 1.6 to 4.0 days accounts for the mixing and distribution of the newly synthesized labeled cholesterol and includes such processes as esterification and distribution of the labeled sterol from the plasma to the tissue. The dominant slow rate of $t_{1/2}^{a}$ = 15 to 25 days might then describe the turnover of plasma cholesterol after the initial rapid processes have been completed.

Since it is known that liver and red cell cholesterol are in isotopic equilibrium with plasma cholesterol (3, 6), a calculation can be made

of the amount of activity incorporated as cholesterol in these organs There are approximately 3 g of free cholesterol present in red cells 3 8 g of free cholesterol and 0 7 g of ester cholesterol contained in liver Using these quantities approximately 3% of the activity administered as acetate has been incorporated as cholesterol in plasma red cells and liver This figure represents a minimum value for cholesterol synthesis since there are undoubtedly pools other than those mentioned

The amount of C^{14} incorporated from acetate into cholesterol varies as much as 4 fold from patient to patient This may be explained by the fact that cholesterol produced in the body from acetate represents a very minute fraction of the total utilization of the 2 carbon precursor the major portion of the acetate is metabolized excreted or utilized in the biosynthesis of other body constituents Therefore the amount of radioactive acetate incorporated into cholesterol depends on the quantity of the administered dose that escapes utilization by metabolic processes other than cholesterogenesis Since these transformations consume by far the overwhelming majority of the administered acetate cholesterol synthesis may vary widely depending on the amount of radioactive acetate metabolized by all the other processes

The Incorporation of Dietary Cholesterol into Plasma Cholesterol

Figure 2 shows the specific activity curves of plasma free and ester cholesterol following the oral administration of cholesterol 4 C^{14} The plasma free cholesterol radioactivity originating in the diet (exogenous incorporation) is at a minimum value with the 3 hour sample The specific activity then rises to a peak at about 1 5 to 2 days and afterwards slowly declines The radioactivity of the "exogenous plasma ester cholesterol is also at a minimum with the first sample rises less rapidly than that of the free sterol and reaches a peak value at about 2 5 days intersecting the free curve at this point After this crossover point the ester specific activity is greater than that of the free for the duration of the period of experimental observation

From Figs 1 and 2 a comparison can be made of the rates of decline of endogenously and exogenously derived cholesterol With the exception of the differences in specific activity during the early portions of the experiment which have been described above it will be noted that the radioactivity of free and ester cholesterol whether derived from the diet or made *in vivo* declines at a similar rate during the 30 day period of observation

The absorption of the fed cholesterol 4 C^{14} varies from 5 to 80% The subjects in this study received approximately 10 mg of labeled

material and the efficiency of absorption may be related to the amount of administered labeled material compared to the quantity of cholesterol in the diet The fractions of the fecal radioactivity derived from cholesterol-4 C^{14} which had been absorbed metabolized and subsequently excreted and that which can be attributed to administered sterol which completely escaped absorption are not known Since only 0 3 to 3 6% of the cholesterol 4 C^{14} appears in the urine the kidney is a minor route for the total excretion of the steroid nucleus

After the feeding of labeled cholesterol from 4 to 14% was present in the circulation at the maximum specific activity of free cholesterol

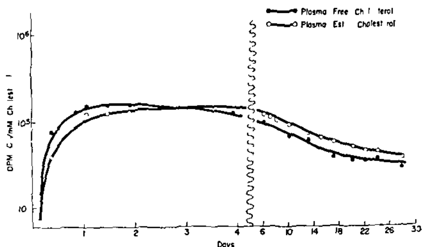


FIG 2 The specific activity curves of plasma free and ester cholesterol following the oral administration of cholesterol-4 C^{14}

It can be calculated that approximately 8 to 33% of the administered dose is present in the liver and circulation this represents a significant fraction of the amount of labeled sterol absorbed from the diet

In several patients in whom expired air collections were made no labeled CO was detected after the administration of cholesterol-4 C^{14} This shows that the A ring of the steroid nucleus and probably the entire ring system remained intact and suggests that compounds having the steroid nucleus can be eliminated from the body only in an intact form either through the urine feces or skin

The rates describing the decline of plasma cholesterol derived from cholesterol-4 C^{14} are remarkably similar to those representing the decline of plasma cholesterol formed by endogenous synthesis from acetate 2 C^{14} (3) Either labeled cholesterol or labeled acetate may be used interchangeably to trace the decay curve of plasma cholesterol pro

vided it is recognized that the early portions of the specific activity curves derived from these two tracer materials are different

These data suggest that dietary cholesterol and cholesterol synthesized in the body mix indistinguishably after the initial processes of absorption and distribution have been completed If the body distinguishes between endogenous and exogenously derived cholesterol this could seemingly occur only during the immediate period after absorption or synthesis These conclusions have been experimentally confirmed in patients who received C^{14} labeled acetate and tritium labeled cholesterol simultaneously

Absorption of Dietary Cholesterol

Absorption of cholesterol via the intestinal lymphatics in the rat dog *and rabbit accounts for essentially all of the sterol absorbed by these* animals from the diet. However steroid hormones are absorbed via the portal system in human subjects (1) It was therefore of interest to study the route of absorption of cholesterol in man

Cholesterol-4 C^{14} was administered orally to a patient in whom a cannula had been previously placed in the thoracic duct The lymph flowing from the cannula was continuously collected so that fractions corresponding to predetermined intervals of time could be obtained (4)

The peak specific activity of absorbed cholesterol in lymph was attained approximately 4 to 5 hours after the administration of the dose as shown in Fig 3 From 7 to 14% of the labeled cholesterol had been absorbed into the lymphatics which lead to the thoracic duct and of the cholesterol absorbed about half was esterified The quantity of labeled cholesterol which appeared in plasma was usually only 1 to 10% of the amount in the lymph This is in accord with the left thoracic duct functioning as the principal route by which cholesterol is transported from the intestine

The specific activity of lymph C^{14} ester cholesterol derived from dietary sources is equal to or greater than the specific activity of lymph free cholesterol especially during the early phases of the experiment Analysis of lymph cholesterol at various intervals during the experiment showed that 50 to 60% of the sterol was esterified These facts indicate that 50 to 90% of the labeled dietary cholesterol is esterified in the intestinal mucosa

From studies carried out in intact subjects it is known that when cholesterol 4 C^{14} is fed free cholesterol appears in the plasma first and esterified cholesterol some time later The maximum radioactivity in the circulation is not attained until 24 to 48 hours after the administration of the dose Where free cholesterol C^{14} was fed to a patient with

a lymph cannula the major portion of labeled cholesterol in the lymph was esterified These data concerning lymph and plasma ester cholesterol can be reconciled by assuming clearance of newly absorbed cholesterol esters from the circulation The participation of the liver in the clearance of cholesterol esters from the circulation is likely The results from this laboratory concerning lymphatic transport of cholesterol in the human are similar to previous experiments in animals and support

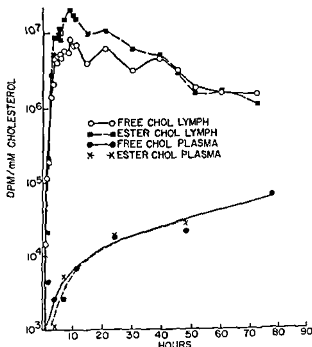


FIG 3 The specific activity curves of lymph and plasma free and ester cholesterol following the oral administration of cholesterol-4 C^{14} to a patient with a thoracic duct cannula in place

the concept of the importance of esterification of cholesterol during the absorptive process

Fecal Sterol Metabolism

Studies such as those previously described as well as sterol balance studies carried out in these laboratories permitted the accumulation of data concerning the quantity and types of fecal steroids excreted by subjects with normal and elevated plasma cholesterol levels Table I lists these values for normocholesteremic subjects on a hospital diet Besides the substances listed the nonsaponifiable fraction also includes some oily "hydrocarbon material and some "more polar" substances

which are probably steroidal An investigation of the hydrocarbon fraction failed to detect the presence of squalene It can be seen that the largest variation appears in the coprostanol content with less variability in the fecal cholesterol

TABLE I
FECAL STEROID EXCRETION IN SUBJECTS WITH NORMAL PLASMA CHOLESTEROL LEVELS[a]

Substance	Approximate weight range (mg/day)
Coprostanol	500–2000
Cholesterol	100–200
Coprostanone	100–200
Cholestanol	25

[a] The nonsaponifiable fraction of feces amounts to 1000 to 3000 mg/day

In a normal subject who was maintained on a cholesterol free fat free diet for 4 days the fecal excretion of sterols amounted to approximately 500 mg per day Assuming a steady state this figure represents the minimal daily turnover of the body cholesterol pool If the cholesterol pool including liver and blood cholesterol is 13 5 g it can be calculated that the turnover time of such a pool is 27 days and that the half life is about 19 days This value obtained by independent means is in good agreement with the half life ($t_{\frac{1}{2}}^{a}$) of plasma cholesterol of 15 to 25 days which was derived from the fall-off curve of either endogenously synthesized or orally administered radioactive cholesterol in experiments carried out over a 30 day period (3)

Either plasma free or ester cholesterol could serve as the immediate precursor of fecal sterols Fecal sterols are derived from a pool which is in isotopic equilibrium with plasma cholesterol (5 7) Although esterified cholesterol could not be isolated from feces this does not preclude it from being the immediate precursor of fecal sterol since hydrolysis could have occurred in the intestinal lumen

Since coprostanol represents the largest single constituent of the nonsaponifiable fraction of human feces and since it is not present in the diet the mechanism of the formation of coprostanol was investigated Figure 4 shows two possible routes of formation of coprostanol The upper portion (A) illustrates the direct conversion of cholesterol to coprostanol by hydrogenation of the double bond without oxidation of the hydroxyl group and the lower portion (B) represents a conversion where Δ^4 cholestenone is an intermediate Experiments from our laboratories utilizing deuterium and C^{14} labeled cholesterol indicate that it is likely that the major portion of coprostanol in feces is derived from cholesterol by path (A) since the deuterium was preserved at C 3

whereas it would have been lost if the route (B) involving cholestenone had been utilized (8 11)

Regulation of Plasma Cholesterol Level by Fecal Sterol Excretion

The etiology of elevated plasma cholesterol levels involves considera tion of many mechanisms among which are increased cholesterol syn thesis decreased cholesterol degradation including decreased conver sion to other metabolites such as bile acids and decreased loss of cho lesterol per se from the body Another mechanism involves alterations in the cholesterol gradient between the tissues and the circulation so that

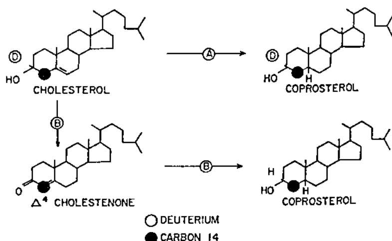


Fig 4 Two possible routes of conversion of cholesterol to coprostanol

the quantity of extracirculatory body cholesterol remains essentially unchanged but the concentration and total quantity present in the plasma are increased In dealing with problems of this sort in intact subjects it is difficult to evaluate changes in synthesis or degradation during the steady state or when a stable level of plasma cholesterol has been achieved However if the level of cholesterol can be conveniently lowered or raised it is possible to devise experimental procedures which permit study of the transition to either the lower or higher level Re duction in excretion during the transition phase could conserve cho lesterol to produce elevation in blood cholesterol or conversely loss of increased amounts of cholesterol could produce reduction in blood levels In view of these considerations the following experiment was designed (7)

A male patient with hyperlipemia and hypercholesteremia was sub jected to alterations in diet which from prior experience would be

expected to change the level of his plasma cholesterol When such an individual is placed on a synthetic diet containing 40% of calories as corn oil the plasma cholesterol usually falls When an isocaloric and otherwise similar diet but containing 40% butter oil calories is sub stituted the plasma cholesterol rises In order to evaluate the events occurring during these dietary manipulations which would account for the expected changes in plasma cholesterol levels the body pool of steroid was labeled by the intravenous administration of radioactive cholesterol 4 C^{14} From prior work (3) it was known that this labeled cholesterol is excreted with the ring system intact although *in vivo* alterations in the side chain and functional groups on the ring do take place Since the radiocarbon atom remained in the steroid molecule throughout all chemical transformations as well as excretory processes it provided a means by which the total steroid excretion could be ac curately measured The presence of carbon radioactivity then directly reflected the presence of intact steroid molecules In order to relate the radioactivity measured in the feces to the weight of fecal steroid the standard procedure of isotope dilution was employed If the fecal radioactivity excreted during a day (disintegrations per minute DPM) is divided by the radioactivity present in a milligram of serum cho lesterol (DPM/mg) on that day the result gives the number of milli grams of serum cholesterol which would be required to contribute that amount of radioactivity to the feces By this calculation and the as sumption which was later experimentally verified that plasma and fecal sterols have the same specific activity it is possible to determine the total amount of fecal steroid excreted during the periods of dietary manipulation when plasma cholesterol levels were either rising or fall ing This entire procedure may be termed a "labeled steroid balance study

It is necessary to study only the fecal excretion since the urinary excretion of approximately 20 mg per day is negligible when com pared to fecal excretion of several grams per day It is not possible to carry out this type of experiment satisfactorily by the direct chemical determination of the fecal steroids because the sitosterols present during the corn oil period cannot be chemically distinguished from cholesterol Likewise the methods for determining the excretory products of bile acids in human feces are not satisfactory and yield only approximate results

The specific activities of plasma and fecal sterols were determined after appropriate radiochemical purification by techniques we have de scribed previously (8 9)

Tracer doses of cholesterol 4 C^{14} were given intravenously at two

points during the experiment once at the very beginning during an *ad lib* dietary adjustment period and again after a stable plateau of plasma cholesterol levels had been obtained on the corn oil diet The actual plan of the experiment including the various dietary periods extending over 6 months is shown in Fig 5

As shown in Fig 5 the plasma cholesterol level fell from about 750 mg % to approximately 500 mg % during 2 weeks when the subject was on an *ad lib* hospital diet When the diet was changed to the experimental 40% butter oil formula, the cholesterol levels rose to approximately 750 to 800 mg % and remained there until an isocaloric

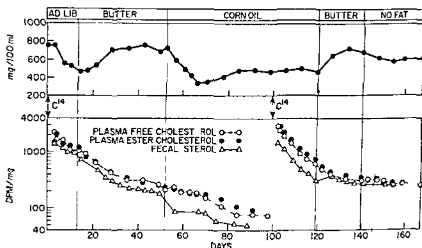


FIG 5 The specific activity and concentration of plasma cholesterol during a labeled sterol balance study

40% corn oil was substituted for butter The corn oil produced a sharp fall in cholesterol levels to about 350 mg % followed by a rise to a stable level of about 500 mg % This level was maintained until 40% butter oil was substituted for the corn oil, and the plasma cholesterol rose again to about 750 mg % The last portion of the experiment was a period in which a diet devoid of fat or steroid was administered and some decrease in cholesterol level occurred during this period The radioactivity of fecal sterol during the period when the diet contained no fat or sterol was the same as that of plasma sterol thereby directly confirming the validity of the isotope dilution calculation discussed previously During the corn oil period however sitosterol derived from the corn oil was excreted in the feces and since it is virtually inseparable from cholesterol it diluted the radioactivity of excreted sterol so that the specific activity of fecal sterol was less than that of the plasma

About 50% of the administered dose was excreted within 30 to 40 days This finding is in reasonable agreement with our previous estimates of cholesterol half lives in the human obtained from the analysis of the slopes of plasma cholesterol specific activity curves following administration of either sodium acetate 2 C^{14} or cholesterol-4 C^{14}

Fecal cholesterol and coprostanol represented the largest portion about two thirds of the excreted radioactivity The remaining third was composed of acidic material presumably derived from bile acids The quantitative nature of the extraction procedures is well illustrated

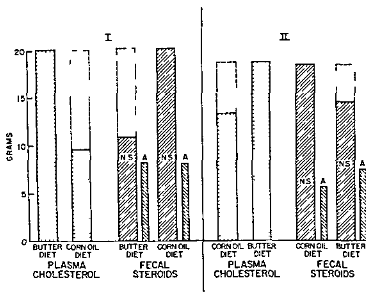


FIG 6 The changes in plasma cholesterol and excretion of fecal sterols in response to changes in dietary lipid

by the fact that the sum of the radioactivity of the individually isolated acidic and nonsaponifiable fractions was equal to the total fecal radioactivity as determined independently

Figure 6 illustrates the relative changes in the plasma and fecal sterols in response to the dietary changes Two periods or transition phases are illustrated during which these comparisons were made the left hand portion (I) where the rapid fall in plasma cholesterol took place when the diet was switched from butter to corn oil and the right hand portion (II) where the plasma cholesterol again became elevated on returning to the butter diet

The total amount of cholesterol in the plasma was determined by multiplying the plasma cholesterol concentration by the plasma volume

The quantity of fecal steroids was determined in each collection by the isotope dilution method The difference between the total sterol excretion during each transition period and the sterol excretion during the immediately preceding equilibrium state will be equal to the net retention or loss of cholesterol and all of its transformation products To insure meaningful comparisons, the excretions occurring during the transition phase and the immediately preceding steady state were determined in an identical fashion over equivalent time intervals

As shown in Fig 6 switching from the butter diet to the corn oil diet produced a drop in circulating cholesterol to half its initial value approximately from 20 to 10 g This is illustrated by the first (dotted) set of large bars During this transition there was an increased excretion of fecal sterol approximately equal in quantity to the fall in plasma cholesterol This is illustrated by the second (cross hatched) set of large bars

There was no change in the excretion of acidic material all the increased excretion occurred in the form of coprostanol and cholesterol The absence of any change in acidic excretion is shown by the two small bars labeled A

The right hand portion of Fig 6 illustrates the reverse procedure of switching from corn oil to butter The plasma cholesterol pool increased and this was accompanied by an almost equivalent decrease in the amount of fecal sterol excreted

The data presented demonstrate that declines in plasma cholesterol levels are accompanied by increased fecal excretion of sterol and conversely, that elevations of plasma cholesterol may be accompanied by diminished fecal sterol excretion Other considerations of these data suggest that increased amounts of cholesterol may be present only in the plasma and that the quantity of extracirculatory cholesterol may be normal in hypercholesteremic states The cholesterol gradient between the tissues and the circulation may be changed only on the circulation side of the gradient The plasma cholesterol level may be controlled either by retention of sterol or by the increased excretion of sterol through the intestines

These findings focus attention on the intestine both with respect to regulation of the plasma cholesterol level as well as being the possible site of a metabolic defect in disturbances of cholesterol metabolism

Role of Cholesterol Esterification in Intestine in Relation to Cholesterol Absorption and Excretion and Regulation of Plasma Cholesterol Levels

From a variety of different experimental approaches evidence has accumulated suggesting the central role of cholesterol esterification in the intestine in relation to hypercholesteremic states

Hypercholesteremic patients following an oral tracer dose of cholesterol exhibit an initial reversal for a few hours only of the specific activities of plasma free and ester cholesterol as compared with normocholesteremic subjects (3) We have shown that normocholesteremic subjects maintain a constant specific activity difference between plasma ester and free cholesterol for months following tracer doses of either labeled cholesterol or acetate the ester cholesterol being higher This specific activity difference is abolished in hyperlipemic hypercholesteremic patients both free and ester cholesterol have the same specific activity

We have also shown that the bile acids and bile cholesterol arise from a precursor pool that has the same specific activity as the ester cholesterol of plasma rather than the free sterol (2 10) Adrenal cholesterol ester functions as the precursor of the steroid hormones The cholesterol in atherosclerotic plaques is chiefly ester

These evidences of disturbances in cholesterol ester metabolism in hyperlipemic states as well as the role of cholesterol ester as the precursor of other compounds of physiologic importance indicate the need for further investigation of the influence of the ester moiety on the subsequent metabolism of the cholesterol molecule This approach is strongly suggested also from the observations reported here on the effect of corn oil in enhancing intestinal excretion of cholesterol and of butter oil in reducing fecal loss of cholesterol The possibility exists that the chemical nature of the fatty acid moiety available in the intestine for esterification determines whether the particular cholesterol ester formed can make the transition into the lymph and hence into the body or whether it is unable to pass into the lymph and therefore is available for excretion

References

1 Hellman L Bradlow H L Frazell E L and Gallagher T F *J Clin Invest* 35 1033 (1956)
2 Hellman L Rosenfeld R S Eidinoff M L Fukushima D K Brunschwig A and Gallagher T F *J Clin Invest* 32 573 (1953)
3 Hellman L Rosenfeld R S Eidinoff M L Fukushima D K Gallagher T F Wang C I and Adlersberg D *J Clin Invest* 34 48 (1955)
4 Hellman L Rosenfeld R S and Frazell E L *J Clin Invest* 37 900 (1958)

5 Hellman L Rosenfeld R S Fukushima D K Gallagher T F and Dobriner K *J Clin Endocrinol and Metabolism* 12 934 (1952)
6 Hellman L Rosenfeld R S and Gallagher T F *J Clin Invest* 33 14? (1954)
7 Hellman L Rosenfeld R S Insull W Jr and Ahrens E H Jr *J Clin Invest* 36 898 (1957)
8 Rosenfeld R S Fukushima D K Hellman L and Gallagher T F *J Biol Chem* 211 301 (1954)
9 Rosenfeld R S Hellman L Considine W F and Gallagher T F *J Biol Chem* 208 73 (1954)
10 Rosenfeld R S Hellman L and Gallagher T F *Federation Proc* 14 271 (1955)
11 Rosenfeld R S Hellman L and Gallagher T F *J Biol Chem* 222 3?1 (1956)

Discussion

KATZ Dr Hellman please amplify your statement that hormonal effects were shown to operate on this mechanism

HELLMAN There are data from patients with the malabsorption syndrome which indicate that hydrocortisone can restore cholesterol absorption to a more normal level

HOLMAN I would like to ask two questions (*1*) how did you manage to get patients to allow the cannulation of the thoracic duct? and (*2*) would you care to amplify your statement that these studies having to do with adsorption blood levels and particularly excretion do not necessarily apply to the arterial wall?

HELLMAN In answer to the first question the patients had neoplasms in the head and neck region The cannula was inserted with permission in those patients who were found to be inoperable at the time a definitive surgical procedure was attempted The cannula can be removed by pulling it through the skin incision We have not had any thoracic duct fistulas or other difficulties I would rather defer answering your second question since I think it would unduly prolong the discussion at this moment

STAMLER I have some detailed questions First with reference to some of the exquisite studies on cholesterol metabolism in the thoracic duct fistula patients there is a suggestion that some other lymph channels are continuing to absorb some cholesterol Would you care to comment on the possibility that this is direct absorption into the portal circulation rather than by way of a lymph channel? Second I think you implied that the cholesterol so absorbed stays in the circulation *This would contradict what may be an erroneous idea on my part that in the* course of circulation some is removed by the liver Would you dilate on this? Third are there any studies on the effect of diet on bile acid secretion other than the Cornell butter studies? For example on starch versus glucose which I believe Dr Stare has shown in animals will influence the excretion Finally would you care to comment on the source of the fecal cholesterol and particularly the notion that this represents the product from sloughing intestinal mucosa? Your data would tend to indicate that this might be erroneous since such definite changes occur when going from corn oil to butter It is hard to visualize how intestinal mucosa might be sloughing at variable rates This would suggest perhaps that there is a source of cholesterol other than bile that there is an active intestinal secretion of cholesterol

HELLMAN In patients with complete bile fistulas no cholesterol can enter the gut except by secretion or sloughing of the intestinal mucosa When such patients are given acetate and synthesize labeled cholesterol the fecal sterols have the same specific activity as the plasma sterols The data just presented show that the fecal sterol specific activity is the same as the plasma sterol specific activity Therefore either the intestinal mucosa which sloughs off is in isotopic equilibrium with the plasma sterol or the fecal sterol represents material which has been directly secreted through the intestinal wall

With respect to the other question I meant to imply that while absorption is in progress the sterol radioactivity attained in the plasma may represent in some patients the values which would result if all the newly absorbed cholesterol were retained in the plasma for a short time Of course the material which is in the plasma eventually distributes itself

As far as the other lymphatic channels are concerned there are a number of studies which demonstrate that the left thoracic duct is not the only lymphatic pathway from the intestine In approximately 30% the major duct is on the right side and in many subjects there are right and left ducts We cannot positively exclude absorption through the portal circulation but this does not seem likely

BOYD I should like to ask Dr Hellman whether in the experiments on the hypercholesteremic hyperlipemic subjects there was any abnormality detected in the fatty acid fraction of the cholesterol ester

HELLMAN We did not study the fatty acid moieties of the cholesterol esters although this approach is suggested since the type of fatty acid in the diet seems to influence the subsequent metabolism of cholesterol

GOULD With reference to the question of the origin of fecal sterols we observed in dogs given labeled cholesterol in protein form that cholesterol in the whole intestine reached equilibrium with that in the blood with a half time of about 1½ days Any fecal cholesterol derived from the intestine would be expected to have the same specific activity as that in the blood except during the first few days after administration of a single dose of labeled cholesterol

BYERS To Dr Stamler s question about whether there might be absorption through the portal vein we had opportunity to collect blood from the portal vein at the same time that we collected lymph from the thoracic duct in a patient who bore two plastic catheters simultaneously During the 12 hours following administration of 40 μc of cholesterol C^{14} there was no radioactivity in the portal vein blood This work was done in collaboration with Drs Howard Bierman and Keith Kelly at the City of Hope Hospital in Los Angeles Their technique for catheterizing the thoracic lymph duct eliminates all cross channeling Under these conditions there was also no appearance of radioactivity in the patient s peripheral blood

BERGSTROM I have just one comment on this paper There might be at least two different mechanisms by which this dramatic change took place The amount of cholesterol in the bile might have increased markedly or the drastic change in the fatty acid composition might have changed the micelle formation in the intestine and hence the cholesterol absorption Do you have any data as to the bile cholesterol? The cholesterol in the bile increases 3 times in the hyperthyroid fistula rat as was first found by Friedman *et al* As the normal cholesterol content of the bile daily secreted in the human is 1 or 2 grams this might be a factor of importance

SAMUELS The chairman feels that the purpose of a conference such as this is to bring the basic observations in relationship to clinical findings In Dr Popjak's presentation the importance of triphosphopyridine nucleotide in reduced form in cholesterol synthesis was brought out very clearly Now as we have gone along we have seen that single cells in the aorta and in the arterial tree could synthesize cholesterol and phospholipids and that a fall in blood pressure as Dr Werthessen indicated increased the synthesis I would like to point out that there is a definite competition between two important systems the cytochrome oxidase system and those systems involved in the steroid hydroxylations This can be demonstrated by the introduction of cyanide at low concentrations the action on steroids is then greatly increased I wonder if the reduction in the competition of the cytochrome oxidase system due to reduced oxygen consumption can be an important factor in endogenous formation of cholesterol perhaps ultimately the lipid plaques that we have heard about tonight This might be a unifying point

Influences of Thyroid Pancreatic, and Adrenal Hormones on Lipid Metabolism and Atherosclerosis in Experimental Animals

J STAMLER,[1] R PICK AND L N KATZ

Cardiovascular Department Medical Research Institute Michael Reese Hospital Chicago Illinois

The cardinal problems concerning us at this meeting are (*a*) the general problem of the etiology and pathogenesis of atherosclerosis and (*b*) the specific problem of the role of hormones if any in the pathogenesis and etiology of atherosclerosis In this presentation our discussion of these problems is based preponderantly on our animal experimental work No attempt is made to review the literature or analyze the available information from clinical pathologic research on man (5 6)

This paper endeavors to focus on three questions (*1*) Under what conditions have definite hormonal effects on experimental atherogenesis been demonstrated? (2) What are these effects? and (3) From the available findings what conclusions can be drawn at this juncture concerning the role of hormones in the pathogenesis and etiology of atherosclerosis?

In approaching the first question a retrospective look at historical experience in atherosclerosis research is most illuminating (4 5) The fact is that over the years many experiments have been carried out on the effects of the endocrines on the arteries Among other findings these studies have shown that a number of hormones—adrenalin thyroid insulin posterior pituitary parathyroid—are capable of producing vascular damage i e *arteriosclerosis* when administered exogenously The remarkable fact clearly emerges however that these early experiments uniformly failed to produce *atherosclerosis* (4 5) By and large the vascular lesions were of the medial degenerative calcific variety with or without intimal hyperplasia and fibrosis (depending apparently on the time dose course of hormone)—but never with atherosclerosis To our knowledge atherosclerosis has never been produced on any experimental animal solely by hormonal manipulation[1]

The impact of this conclusion can be fully appreciated only when

[1] Established Investigator American Heart Association 195—8 as of April 1 1958 Director Heart Disease Control Program Chicago Board of Health

[1] The single exception is the production with estrogens in cockerels of sustained endogenous hypercholesterolemic hyperlipemia leading eventually over many months to aorta (but not coronary) atherosclerosis (5) This occurs in cockerels on a plain mash diet devoid of a cholesterol supplement Estrogens inhibit coronary (but not aorta) atherogenesis in chicks on a high cholesterol diet—cf the paper presented by Dr Ruth Pick at this Conference

detailed attention is given to the design of these early experiments They uniformly involved administration of hormones to animals subsisting on their usual diets That is in none was the diet supplemented with fat and cholesterol Under these circumstances no influences of hormones on atherogenesis can be demonstrated Therefore it would seem valid to amplify our conclusion Hormonal derangements are apparently incapable of inducing atherosclerosis in the absence of an atherogenic diet This would seem to be an inexorable conclusion based on the early work viewed from present day vantage points

The findings of the modern era have served to round out the picture They have clearly shown that hormones are indeed capable of exerting very marked influences on atherogenesis in animals ingesting potentially atherogenic diets—but only under those circumstances Therefore most of the modern work on hormones and atherosclerosis deals with the effects of hormones in animals ingesting a potentially atherogenic diet Most of our experiments over the last decade—with few exceptions—have been of that type One of the few exceptions is illustrated below since it highlights the point made and demonstrates one or two other significant facts (cf Fig 18)

We may now proceed to question No 2 reformulating it—based on the foregoing—to read What effects of hormones on atherogenesis have been observed in animals on potentially atherogenic diets? Practically all of the studies undertaken to answer this question have had their origin in well known problems of clinical medicine Thus thyroid hormone was one of the first to be explored experimentally—based on the numerous leads from clinical investigation suggesting interrelationships among thyroid cholesterol metabolism and atherogenesis Many years ago Turner Page Bernhard and others showed that administration of thyroid hormone suppressed hypercholesterolemia and atherogenesis in cholesterol fed rabbits (5 8 26) More than 10 years ago similar observations in chicks were made in our department See Fig 1 (2) Thyroid hormone partially suppressed cholesterol induced hypercholesterolemia and atherogenesis Potassium iodide was without influence It was later shown that orally administered desiccated thyroid powder was also capable of partially inhibiting endogenous estrogen induced hyperlipemia and aorta atherogenesis in cockerels on plain mash diets devoid of a cholesterol supplement (18)

Subsequently an experiment was undertaken to evaluate whether the effect of thyroid was a by product of hypermetabolism For this purpose a comparison was made of hypermetabolism induced by thyroid feeding and by dinitrophenol in chicks on a high fat, high cholesterol ration See Fig 2 (24)

TABLE I

…s of Various Thyroid Preparations on Plasma Lipids and Atherogenesis in Cockerels on High Cholesterol High Fat Diets (S 29 11/53–12/53 5 Weeks)

	Survival to end of experiment (%)	Feed intake (g /chick/day)	Terminal weight (g)	Plasma total cholesterol[a] (mg %)	C/P ratio[b]	Gross thoracic aorta atherogenesis (incidence and grade)		Microscopic coronary atherogenesis (incidence and extent)	
	100	107 ± 12	1683 ± 69	384 ± 56	1 31 ± 0 19	90%	1 0 ± 0 2	100%	15% ± 2
	90[c]	119 ± 16	1303 ± 33	287 ± 27	1 58 ± 0 15	67%	0 8 ± 0 2	33%	10% ± 1
…ne	*60[d]*	*144 ± 29*	—	*191 ± 11*	—	83%	0 5 ± 0 1	83%	12% ± 5
…yrosine	100	107 ± 11	1218 ± 50	248 ± 15	1 01 ± 0 09	70%	0 8 ± 0 3	100%	10% ± 2
…hyronine	90	110 ± 10	1348 ± 63	337 ± 35	1 36 ± 0 07	89%	0 8 ± 0 2	100%	10% ± 2
	90	110 ± 15	1484 ± 40	529 ± 38	1 35 ± 0 09	100%	1 6 ± 0 2	100%	22% ± 5

…ata from 2 bleedings at 2 and 5 weeks on experiment except for thyroxine group which was bled at 2 weeks only

…ata from final bleeding only

…ages Desiccated thyroid powder—1 0% orally in the mash or 1190 mg /bird/day or 2 3 mg organic iodine daily
Thyroxine—6 mg /chick/day or 3 9 mg organic iodine daily
Diiodotyrosine—3½ cc of a special preparation (RESH) parenterally containing 2 1 mg organic iodine daily
Triiodothyronine—1¼ mg daily or 0 9 mg organic iodine
KI—100 mg /200 cc drinking water

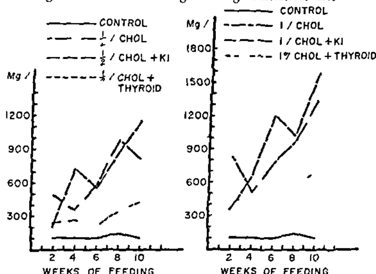
…ree additional birds died 1 day before sacrifice

… additional birds died 1 day before sacrifice

The dinitrophenol treated group exhibited findings similar to those of the birds ingesting an atherogenic mash without a hypermetabolism inducing agent (*groups 1 and 2 Fig 2*), *the thyroid treated group* (group 3) had significantly less hypercholesterolemia organ cholesterosis and aorta atherosclerosis Hence the effect of thyroid apparently was not simply a by product of the hypermetabolism it induced

In the foregoing and subsequent experiments a marked lipotropic

Effects of desiccated thyroid and potassium iodide on cholesterolemia and atherogenesis in chicks on high fat high cholesterol diet

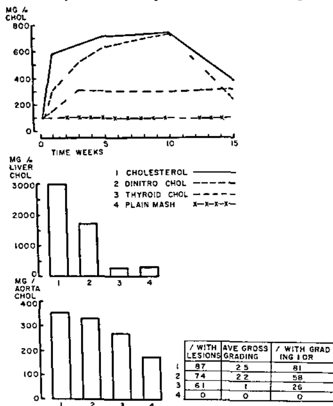


	% th Lesions G d –	% th Lesion G d ~	% with Lesion G ad >3
Choles r l Cont l	33	67	
Ch les er l pl Thyroid	48	5	
Ch lesterol pl KI	3	69	2

FIG 1 Chol is cholesterol incorporated in chick mash at the 0 5 or 1 0% level 20% cottonseed oil was also mixed into the mash The dosage of potassium iodide (KI) was 0 14–0 15% of the diet given in the drinking water by pipette or in the mash respectively in the three series of experiments The dosage of desiccated thyroid powder was 0 02% 0 10% and 0 07–0 08% respectively in *the three series of experiments* The chicks were 5–7 weeks of age at the onset of the experiments The two upper graphs are a plot of plasma total cholesterol levels Gross atherosclerotic lesions were graded on an arbitrary scale from 0 to 4 (2 5)

action of thyroid hormone was demonstrable in cholesterol fed cockerels See Figs 2 and 3 (12 24) In the course of these multiple studies with desiccated thyroid and related preparations (thyroxine diiodotyrosine triiodothyronine) we became impressed as much by the partial in complete nature of their effects as by the effects themselves Experiments with results leading to this interpretation are illustrated in Fig 4 and Table I (21) Thyroid preparations in these experiments tended

Effects of desiccated thyroid and dinitrophenol in cockerels on a high cholesterol diet



	/ WITH LESIONS	AVE GROSS GRADING	/ WITH GRADING 1 OR
1	87	2 5	81
2	74	2 2	58
3	61	1	26
4	0	0	0

Fig 2 Groups 1–3 received 2% cholesterol in the mash one half the birds in each of these groups were also given 5% cottonseed oil in the mash the other half were not given any oil supplement The results were similar with and without cottonseed oil therefore these data for each group were pooled Desiccated thyroid powder was incorporated in the mash at the 0 5% level (group 3) Dinitrophenol was mixed into the mash at the 0 04–0 09% level being given in progressively increasing dosage to the point of gross signs of toxicity with subsequent reduction to the 0 07% level The data on lesions refer to gross atherosclerotic plaques in the abdominal aorta (table in lower right) The three graphs present data on total cholesterol in the plasma liver and aorta respectively (24)

to induce partial inhibition of *hypercholesterolemia* but tended to have little or no effect on aorta or coronary atherosclerosis[2] Findings of this type tended to occur particularly with large doses of the thyroid preparations They suggested that the effects of thyroid here are probably more complex than their influences on cholesterol lipid metabolism This interpretation is supported by abundant data in the older literature indicating that thyroid in large doses is capable of

Lipotropic effect of desiccated thyroid in *cockerels fed* high cholesterol diets

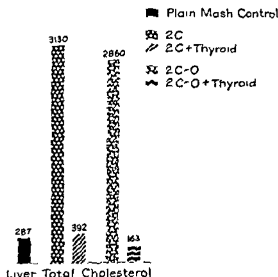


Fig 3 Here and in subsequent figures 2C is mash supplemented with 2% cholesterol 2C O is mash supplemented with 2% cholesterol plus 5% cottonseed oil. Desiccated thyroid powder was incorporated in mash at the 0 5% level Liver total cholesterol values are in mg % (12)

producing vascular damage—i e arteriosclerosis (not atherosclerosis)—in animals not receiving dietary supplements of fat and cholesterol It is therefore possible that the findings of the present experiments (Fig 4 and Table I) represent the net results of opposing tendencies—i e vascular damage from high dosage of thyroid hormone tending to create foci for atherogenesis in cholesterol fed animals and thyroid induced partial inhibition of hypercholesterolemia tending to retard atherogenesis A similar conclusion was reached many years ago based on comparable findings in rabbits (1) In actuality the intertwined

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Note that KI and the thyroid stimulating hormone of the pituitary did not in this or other experiments exert any inhibiting effects on hypercholesterolemia or atherogenesis See Fig 4 and Table I (2 21)

effects are probably even more complex and in all likelihood involve thyroid effects on protein and vitamin (as well as lipid) metabolism—effects which may influence atherogenesis (22 23)

Effects of thyroid and TSH in cockerels on a high fat high cholesterol diet
Series 19 + 21

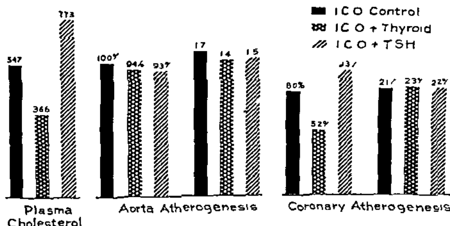


FIG 4 1C-O is mash supplemented with 1% cholesterol plus 5% cottonseed oil TSH is thyroid stimulating hormone of the anterior pituitary Thyroid hormone was given in mash at the 1 0% level TSH dosage was 5 0–25 0 mg /bird/day in S19 and S21 respectively The data on aorta atherogenesis are per cent of birds with gross atherosclerotic plaques i e incidence of lesions (first set of 3 bars) and mean grading of gross lesions graded on an arbitrary scale 0–4 (second set of 3 bars) The data on coronary atherogenesis are per cent of birds exhibiting microscopic atherosclerotic plaques i e incidence of lesions (first set of 3 bars) and mean per cent of involvement by plaques in vessels of individual birds designated the coronary count (second set of 3 bars) The latter value is an index of severity of atherogenesis It is obtained by studying two standardized sections from each heart, counting the total number of arterial and arteriolar vessels visualized the number that exhibit normal morphology lipid infiltration of the vessel wall and plaque formation respectively The coronary count is vessels with plaques/total vessels expressed as per cent Similar data on aorta and coronary atherogenesis are presented in subsequent figures (5 21)

These possibilities are supported by data from another experiment of somewhat different design (Fig 5) All the previous experiments may be designated experiments exploring the *prophylactic* potential of a hormone In them the hormone possibly influencing atherosclerosis is administered during an experimental period coincident with the institution of a potentially atherogenic diet The design of the experiment illustrated in Fig 5 is different This may be designated an experiment exploring the *therapeutic* potential of the hormone An athero

genic diet is first fed for a number of weeks to all groups of birds Then one group is sacrificed to assess the status of lesions The other two groups are returned to a plain mash diet devoid of a supplement of cholesterol and fat In chicks this results in beginning regression of lesions within 2 weeks This regression of lesions is particularly noticeable in the coronary vessels at this time (Fig 5)

Effects of thyroid on diet induced regression of atherosclerosis in cockerels

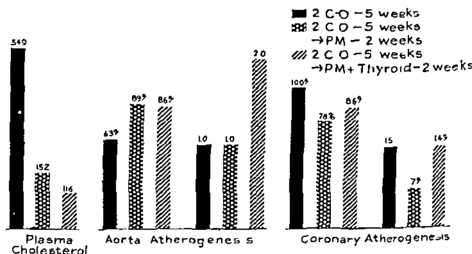


Fig 5 All three groups received 2C O diet during the first 5 weeks Group 1 was then sacrificed Groups 2 and 3 were transferred to plain mash (——→ PM) for 2 weeks (the regression or therapeutic period) and then sacrificed Desiccated thyroid powder was given at the 0 5–1 0% level in this experiment

But when desiccated thyroid was administered with the plain mash diet during these last 2 weeks regression was retarded Thus under these circumstances thyroid did not exert a favorable influence on regression of lesions although in both groups (experimental and control) hypercholesterolemia virtually disappeared particularly in the group on thyroid medication These findings again suggest that large doses of thyroid hormone exert multiple complex effects Further work is proceeding on this problem at the present time

In addition to the many studies on the effects of thyroid hormone administration and hyperthyroidism considerable experimental work has been done on the influence of hypothyroidism This Conference is well aware that the combination of hypothyroidism and cholesterol fat feeding was successfully used by Steiner and Kendall to produce atherosclerosis in dogs (5 25) Similar work has been done in rats Our group

observed that hypothyroidism counteracted the ability of estrogens to inhibit coronary atherogenesis in cholesterol fed cockerels (see the paper presented by Dr Ruth Pick at this Conference)

Over the years our group has also extensively explored the effects of pancreatectomy and pancreatic hormones being stimulated in these studies by the well known observations of intensified atherogenesis in human diabetics male and female (5) An early experiment on this problem is illustrated in Fig 6 (15) Note that diet induced hyper

Effects of pancreatectomy in cockerels on a high fat high cholesterol diet

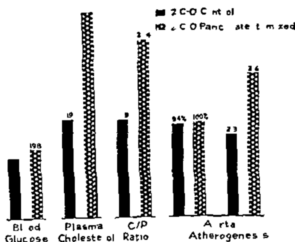


Fig 6 C/P ratio is the ratio of plasma total cholesterol to plasma total phospholipids

cholesterolemia was consistently greater in the pancreatectomized cockerel For those not familiar with the peculiarities of avian physiology it should be noted that the pancreatectomized chick on a plain mash diet grows normally and does not develop hyperglycemia glycosuria hyperlipemia ketosis acidosis To all intents and purposes the depancreatized cockerel—and the alloxanized chick as well—is apparently a normal animal in contrast to the pancreatectomized or alloxanized dog or rat Under special experimental conditions however definite metabolic abnormalities can be elicited in the depancreatized cockerel Thus a markedly enhanced hypercholesterolemia supervened in pancreatectomized chicks fed a mash supplemented with 2% cholesterol and 5% cottonseed oil (Fig 6) With diets of less severe atherogenic potential this abnormality could not be demonstrated (Figs 7 and 8)

The effects of pancreatectomy were also studied in the regression

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Effects of thyroid on diet induced regression of atherosclerosis in cockerels

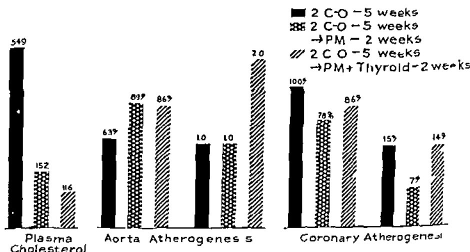


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tive effects of insulin were observed in these cockerels on a high fat high cholesterol diet [3] However an experiment of the therapeutic type revealed that insulin definitely interfered with regression of atherosclerotic lesions in birds transferred from an atherogenic to a normal diet (Fig 11) Further insulin counteracted the ability of estrogens to prevent cholesterol induced coronary atherogenesis—a finding which Dr Ruth Pick discusses more fully elsewhere in the proceedings of this Conference The mechanism of these effects of insulin remains to be elucidated Large doses of insulin were used and the birds were hypoglycemic for periods As a result, patterns of food consumption were affected Almost certainly—as indicated by periods of reactive hyper

Effects of pancreatectomy on diet induced regression of atherosclerosis in cockerels



Fig 9 1C-O ⟶ PM + Sham is a 1C-O diet for 5 weeks sham operation for pancreatectomy then plain mash (PM) diet for 2 weeks (cf Fig 5)

glycemia—hyperinsulism tended to provoke increased secretion of adrenalin and corticoids Whether these complex responses are related to the effects of insulin on atherogenesis is not at all certain at present

These findings with insulin rekindled our interest in data on alloxan diabetic rabbits published a few years ago by some of our colleagues at this Conference (3 7) The alloxan diabetic cholesterol fed rabbit was found by them to exhibit markedly elevated serum cholesterol levels with a normal cholesterol/phospholipid (C/P) ratio and little or no atherosclerosis despite the tremendous hyperlipemia These diabetic rabbits were generally not treated with insulin However in one experi

[3] This is one of the few experiments in our laboratory exploring the effects of adrenalin In the limited way carried out it is an inconclusive study It was long ago shown by others that administration of adrenalin induced arteriosclerosis—but not atherosclerosis—in experimental animals consuming their usual diets devoid of any cholesterol fat supplement (4) It was also shown that adrenalin induced arteriosclerotic plaques acted as sites of predilection for lipid deposition and super imposed atherogenesis in rabbits on high cholesterol diets (1)

therapeutic type of experiment (Fig 9) In the early regression period pancreatectomy apparently interfered with regression of both hyper cholesterolemia and atherogenesis These findings suggest that an intact pancreas plays a role in effecting regression of hypercholesterolemia and atherogenesis in cockerels transferred from an atherogenic to a normal diet

These observations in depancreatized chicks led to studies with in sulin A prophylactic type experiment is illustrated in Fig 10 no defini

Effects of pancreatectomy in cockerels fed various diets—weeks 7 22

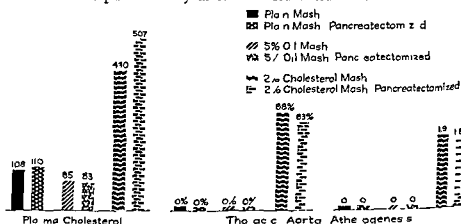


FIG 7 5% oil mash is mash supplemented with 5% cottonseed oil (5 15)

Effects of alloxanization and pancreatectomy in cockerels fed a high fat high cholesterol diet—weeks 13 21

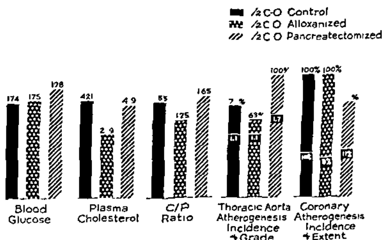


FIG 8

no increase in C/P ratio (since phospholipids tended to rise parallel with cholesterol under the influence of hydrocortisone) and little or no aggravation (or amelioration) of atherogenesis (Fig 12) (9 20) Similar results were obtained with adrenocorticotropic hormone (ACTH) (Fig 13) On a diet of 0 25% cholesterol and 5% cottonseed oil a control group of cockerels receiving no hormone exhibited minimal hyper cholesterolemia and no atherosclerosis in experiments of 5–15 weeks duration When birds on such a diet were given hydrocortisone in

Insulin counteraction of diet regression of cholesterol induced atherosclerosis
Series 33 and 38

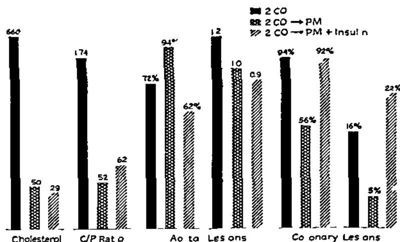


Fig 11 Insulin dosage during the regression period was 5–15 units of a long acting preparation

tensification of both hypercholesterolemia and atherosclerosis was observed (Fig 14) (9 20) This set of experiences incidentally illustrates the need to explore varying quantitative combinations of hormone and diet in experiments of this type

In agreement with a previous observation utilizing adrenal cortical extract (19) it was further observed that hydrocortisone induced marked hyperglycemia in cockerels Thus diabetic chicks—corticoid diabetic—were for the first time available for study In an attempt to explore their response patterns further hydrocortisone administration was combined with alloxanization and/or pancreatectomy (Fig 15) (9 20) It had been previously shown that alloxanized or depancreatized cockerels responded to glucocorticoids with more marked hyperglycemia than intact controls (19) Although hypercholesterolemia was intensified in these diabetic birds no definitive consistent aggravation of atherogenesis

ment insulin was given and under those conditions significant athero genesis ensued

Such findings in chick and rabbit inevitably pose the question Is it possible that in diabetic persons ingesting diets which might be con sidered potentially atherogenic the therapeutic agent—insulin—might be playing some role in the causation of the aggravated atherogenesis which is such an important feature (complication) of this disease?

Effects of insulin and insulin + adrenalin in cockerels fed a high fat high cholesterol diet Series 43—weeks 10 17 Series 46—weeks 9 14

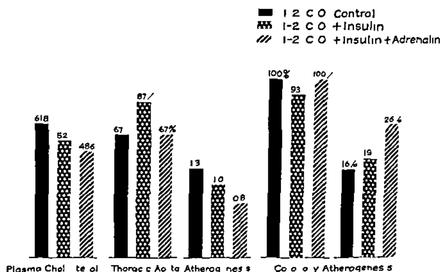


FIG 10 These data are a composite of two experiments yielding similar re sults S43 and S46 with 1C O and 2C O diets respectively The dosage of insulin was 5–15 units of a long acting insulin given as a single daily injection Adrenalin was also given parenterally as a single daily injection of a long acting preparation in oil

To our knowledge no reliable data on man are available bearing upon this problem The experience in experimental animals would suggest that this problem needs to be explored in humans

Another series of experiments was carried out investigating the effects of adrenal corticoids With diets containing a supplement of 5% cottonseed oil and 0 5-2 0% cholesterol cockerels responded to hydro cortisone with marked intensification of hypercholesterolemia[4] little or

[4] In tracer studies carried out in cooperation with Gordon Gould using C^{14} acetate evidence was obtained indicating that this hydrocortisone induced in tensification of lipemia is due at least in part to increased hepatic synthesis of lipid under hormonal influence

glucocorticoid—effects in this avian species (Fig 17) (20) Except in large doses it was generally inactive In large doses it tended to induce a moderate increase in blood pressure In chicks fed a high fat high cholesterol diet this cortisone induced "hypertension" was associated with intensified atherogenesis (Fig 17) (9 20) This finding emphasizes the general problem of the interrelationships among hyper

Effects of ACTH in cockerels fed a high fat high-cholesterol diet—weeks 12–19

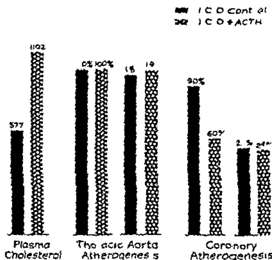


Fig 13 A single daily parenteral injection of a long acting ACTH (10–25 mg) was given (20)

Effects of corticoid diabetes with hyperadrenocorticism in cockerels on a high fat high-cholesterol diet

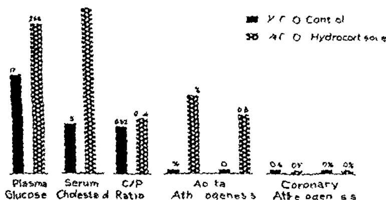


Fig 14

was observed (Fig 15) Here again the rise in plasma cholesterol was paralleled by an increase in phospholipids so that C/P ratios in these diabetic birds were not consistently or markedly different from those in the control group This observation emphasized the possibility that C/P ratio alteration may be a key factor in atherogenesis—a problem discussed in greater detail elsewhere in this Conference in the paper presented by Dr Ruth Pick

Effects of hydrocortisone in cockerels on a high fat high cholesterol diet—weeks 13–21

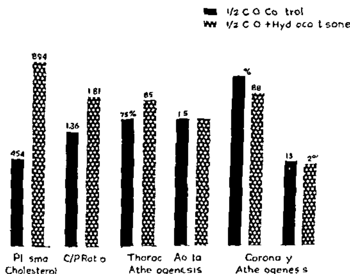


Fig 12 In this and subsequent experiments a single daily parenteral injection of hydrocortisone (1–2 mg) was given (9 20)

A therapeutic type experiment was also accomplished with hydro cortisone (Fig 16) (9) As might have been anticipated from its lipemia inducing effects the hormone delayed regression of hyper cholesterolemia Its administration was also associated with absence of the usual regression of aorta and coronary atherosclerotic plaques usually occurring in birds transferred from an atherogenic to a plain mash diet (Fig 16)

These effects of hydrocortisone—an active glucocorticoid in chicks[5] —are very similar to those of others working with rabbits (cf Dr Ad lersberg s presentation elsewhere in this Conference)

In contrast to hydrocortisone cortisone had androgenic—rather than

[5] Ed note—The principal endogenous corticosteroid in the fowl appears to be corticosterone (Phillips J G and Jones C *J Endocrinol* 16 iii 1957)

tension lipid metabolism and atherosclerosis—a problem forcibly posed by data on man

A more complete experimental exploration of this problem is illustrated in Fig 18 (9 13) It is essential to focus attention on the details of design in the experiment—an experiment analyzing the effects of deoxycorticosterone The first group of cockerels received no hormone and consumed a plain mash diet containing no cholesterol fat supplement The second group ate the same plain mash diet, and re

Effects of cortisone in cockerels on a high fat, high-cholesterol diet—weeks 1-11

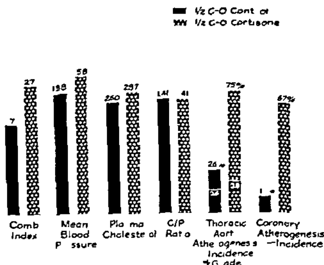


Fig 17 A single daily parenteral injection of cortisone (1–15 mg) was given The dosage was increased step-wise during the course of the experiment. The comb index is a measure of androgenic activity in these growing cockerels (20)

ceived oral salt and parenteral deoxycorticosterone acetate (DCA) to produce an increase in blood pressure (It is a moot question whether this is analogous to human essential hypertension We prefer to speak of it as elevated blood pressure or "hypertension" in quotes) Group 3 ate a mash supplemented with 5% cottonseed oil and cholesterol at the 0.25% level—*a level that effects minimal hypercholesterolemia* and no gross atherosclerosis in experiments of 5–15 weeks duration (gross lesions are observed only after 35 weeks) (14) Group 4 received this same 0.25% cholesterol and 5% cottonseed oil mash, plus salt and DCA Despite the administration of salt and DCA and the resultant increase in blood pressure group 2—eating plain mash—developed no hypercholesterolemia or atherosclerosis This is in accord with multiple ob-

Effects of pancreatic corticoid diabetes with hyperadrenocorticism in cockerels fed a high fat high cholesterol diet

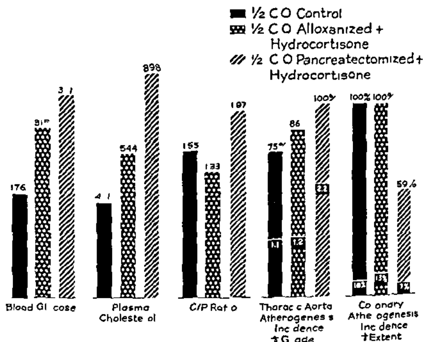


Fig 15

Effects of corticoid diabetes with hyperadrenocorticism on diet induced regression of atherosclerosis in cockerels

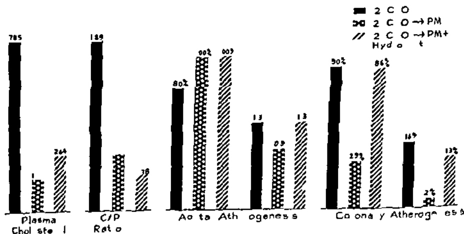


Fig 16

concerning the role of hormones in the etiology and pathogenesis of atherosclerosis?

Based on the available studies exploring the influences of thyroid pancreatic and adrenal hormones (and leaving out of consideration the effects of sex steroids) the following over all conclusions seem justified at this stage of our knowledge

1 In animals consuming their normal diets devoid of fat cholesterol supplements neither ablation of endocrine organs nor administration of excessive doses of hormones induces atherosclerosis Such procedures may cause vascular damage of the arteriosclerotic—but not of the athero sclerotic—variety

2 In animals ingesting potentially atherogenic diets i e diets con taining fat-cholesterol supplements hormonal alterations are undoubt edly capable of influencing atherogenesis

3 These hormonal effects on atherogenesis in cholesterol fat fed animals may be ameliorative or aggravating—depending upon the specific endocrine gland involved the procedure (production of hypo or hyperfunction) the dosage route duration of hormone administra tion etc

4 Multiple observations in a variety of experimental situations in different animals demonstrate clearly that the endocrines significantly influence cholesterol lipid lipoprotein metabolism as reflected by levels of circulating lipids The demonstrable endocrine influences on athero genesis in fat-cholesterol fed animals are almost certainly mediated in part—but only in part—by these endocrine induced effects on lipid metabolism

5 Many observations in animals consuming potentially atherogenic diets indicate that endocrine influences on atherogenesis are not *solely* a resultant of their lipid metabolic effects Among the many hormonal actions that may be playing a role effects on vascular tissue metabolism and on protein and vitamin metabolism need further exploration

6 The totality of findings on dietary hormonal interrelationships in animals—particularly the observed interference of several endocrines with regression of lesions following transfer from an atherogenic to a normal diet—suggests that marked hormonal imbalances may exert an intensifying effect on atherogenesis in the presence of prerequisite diet induced metabolic derangements

ACKNOWLEDGMENTS

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servations in several species—hypertension per se never produces atherosclerosis[6] It produces other types of vascular pathology but never atherosclerosis

When the experimental animal is fed a potentially atherogenic diet the effects of blood pressure elevation are quite different—as has been repeatedly demonstrated in studies on rabbits dogs and chicks (5 6 10 16) The present experiment as already indicated was of too brief a duration for lesions to develop in the group on 0 25% cholesterol and oil without salt and DCA (group 3) However with the additional

Interrelationships between hypertension and atherogenesis in cockerels

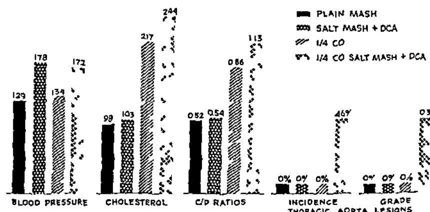


Fig 18 *Sodium chloride (1%) was added to the mash Dosage* of desoxycorticosterone acetate (DCA) was 1–4 mg /bird/day as a single daily parenteral injection dosage was increased step wise during the 15 weeks of the experiment (9 13)

insult of salt steroid administration inducing elevated blood pressure significant gross thoracic aorta atherogenesis occurred in these birds within this time period (group 4) (Fig 18)

When therefore elevated blood pressure is produced in an animal (rabbit dog chick) ingesting an atherogenic diet and exhibiting hyper cholesterolemia as a consequence the result is aggravation of athero genesis

It is now appropriate to summarize and this can best be done by briefly answering the last of the three questions originally posed From the available findings what conclusions can be drawn at this juncture

[6] One special aspect of this problem—the effects of renal disease on both lipid metabolism and blood pressure and consequently on atherogenesis—cannot be dealt with comprehensively in this presentation

25 Steiner A and Kendall F E *A.M.A Arch Pathol* 42 433 (1946)
26 Turner K B *J Exptl Med* 58 115 (1933)

DISCUSSION

STRISOWER In connection with the presentation of data on the effect of potassium iodide in chickens I would like to take this opportunity to mention briefly studies done in humans given potassium iodide Several patients were followed on an out patient basis because of a history of myocardial infarction hypertension or because of xanthoma tendinosum or xanthoma tuberosum All patients had in common elevated serum lipoprotein concentrations in various combinations of S_f^0 classes These patients received potassium iodide 30 mg per day for several months and very careful analysis of the data by statistical means failed to show any effect of potassium iodide on their serum lipoprotein levels

I should like to ask a question of Dr Stamler With respect to his statement that in pancreatectomized chickens insulin produces an increase in hypercholesterolemia and perhaps in atherogenesis but that this effect may be due to diet the slide showed that there was a control but as far as I could tell the control had the same diet I would like some elaboration on this

STAMLER With respect to the experiments on insulin—leaving aside for the moment a finding on estrogen insulin interrelationships which Dr Ruth Pick will discuss later—the observed effects were during the regression period Let me reemphasize the technique Lesions were first produced by feeding chicks a high fat high cholesterol diet for several weeks no hormone was administered at this time Presence of atherosclerosis was verified by sacrifice of one-third of the animals Then the remaining two-thirds of the chicks were divided into two groups both receiving plain mash one receiving insulin the other no hormone Under such circumstances—with the previous atherogenic diet having induced lesions—insulin inhibited the regression of lesions usually seen with restitution to a plain mash diet This inhibition of regression occurred despite the fact that the insulin treated group on plain mash—like the control group—exhibited regression of hypercholesterolemia Therefore the persistence of lesions occurred despite clearing of the hypercholesterolemia

May I just make one other comment on potassium iodide There is an old German saying Wenn mann weiss nicht wie und warum dann gibt man Jod kalium I think we have graduated from that era

BLOCH I would like to ask a very general and presumably naive question Are any of the experimentally induced lesions irreversible? And secondly what is the relation of these experimentally induced lesions to the four stages in the human which Dr Holman talked about?

STAMLER As anticipated Dr Bloch s questions are anything but naive They are very basic questions First of all in every species studied to date—as Anitschkow long ago reported in the rabbit Kendall in the dog and ourselves in the chick—atherosclerosis has been shown to be a reversible lesion at least in part Reversibility of lesions can be virtually complete in young plaques induced in cockerels fed an atherogenic diet for only 5 or 10 weeks Such plaques are characterized by extensive lipid deposition and numerous proliferating fibroblasts without as yet hyalinization scarification calcium deposition cartilage or bone formation All of these latter advanced changes can be induced in the experimental animal by more prolonged feeding of an atherogenic diet The "young lesions in both the aorta

These experiments were carried out over a period of several years with the assistance of the Cardiovascular Department's technical staff in experimental atherosclerosis research including Mrs Dolores Friedman Century Mr Philip Johnson, Mrs Christine Bolene Williams Miss Marilyn Dudley Miss Chizuko Kakita Mrs Eva Levinson Miss Mildred Michael Mrs Eva W Miller Mrs Charlene Thompson Mrs Montez Vankinscott Messrs Grady Crowley John Morris and Ronald Wallace

We are also indebted to Armour and Company Eli Lilly and Company Parke-Davis and Company the Schering Corporation and the Upjohn Company for generous supplies of hormonal preparations for these studies

References

1 Anitschkow N *in* Arteriosclerosis—A Survey of the Problem" Macmillan, New York 1933
2 Dauber D Horlick L and Katz L N *Am Heart J* 38 25 (1949)
3 Duff G L and Macmillan G C *J Exptl Med* 89 611 (1949)
4 Hueper W C *A M A Arch Pathol* 38 162, 245 350 (1944) and 39 51 117 187 (1945)
5 Katz L N and Stamler J Experimental Atherosclerosis C C Thomas *Springfield Illinois 1953*
6 Katz L N Stamler J and Pick R Nutrition and Atherosclerosis Current Status of the Problem (A Monograph) Lea & Febiger Philadelphia Pennsylvania In press
7 McGill, H C Jr and Holman R L *Proc Soc Exptl Biol Med* 72 7? *(1949)*
8 Page I H and Bernhard W G *A M A Arch Pathol* 19 530 (1935)
9 Stamler J Proc Annual Meeting Council for High Blood Pressure Research American Heart Association 1952
10 Stamler J Cerebral Vascular Diseases—Proceedings of the 2nd Conference Grune & Stratton New York In press
11 Stamler J *J Natl Med Assoc* 50 161 1958
12 Stamler J Bolene C Levinson E and Dudley M *Endocrinology* 46 382 (1950)
13 Stamler J and Katz L N *Circulation* 2 480 (1950)
14 Stamler J and Katz L N *Circulation* 2 705 (1950)
15 Stamler J and Katz L N *Circulation* 4 255 (1951)
16 Stamler J Katz L N and Pick R Fourth Annual Report on Stress Acta Inc 1954
17 Stamler J Katz L N Pick R and Rodbard S *Rec Progr in Hormone Research* 11 401 (1955)
18 Stamler J Miller A J Akman L C Silber E N Bolene C and Katz L N *Circulation* 2 523 (1950)
19 Stamler J and Pick R *Proc Soc Exptl Biol Med* 75 803 (1950)
20 Stamler J Pick R and Katz L N *Circulation* 10 237 (1954)
21 Stamler J Pick R and Katz L N *Circulation Research in press*
22 Stamler J Pick R and Katz, L N *Circulation Research* 6 442 (1958)
23 Stamler J Pick R and Katz L N *Circulation Research* 6 447 (1958)
24 Stamler J Silber E N Miller A J Akman L C Bolene C an Katz L N., *J Lab Clin Med* 35 351 (1950)

artery in man are quite peculiar to man as for example the sharp bend of the left descending coronary Now in rabbits I have persistently seen not only atheroma but also thrombosis in the arteries of the stomach after 2 or 3 months of feeding of a cholesterol diet without the addition of fat No necrosis or infarction is seen in the stomach simply because the collateral circulation is good I would like to suggest that we might look at arteries other than the coronaries in experimental animals I think it is perfectly possible that the coronary lesion in man is peculiar to man because of the particular anatomical features rather than because of some peculiarity of the physiological behavior of the coronaries in man

and the coronary vessels can be completely reversed by withdrawal of the atherogenic diet Moreover as Dr Pick will show estrogens will induce regression of plaques in the coronary vessels even when the atherogenic diet continues to be ingested The more advanced lesions—as their histologic structure would indicate—can be induced to undergo partial but not complete regression There is evidence for regression in man too Thus Wilens and Loeber in independent studies both observed less atherosclerosis in a group of persons dying of a debilitating illness compared with an age sex matched group of individuals dying acutely Further *Rivin and Dimitroff found evidence for regression of coronary lesions in men* with prostatic carcinoma treated with large doses of estrogens Time does not permit a discussion of additional evidence from epidemiologic research suggesting regression of atherosclerosis in man

Now with respect to the four morphologic stages of the atherosclerotic lesion in man and their possible counterparts in experimental animals Of course—without going into the matter at length—atherosclerotic plaques in man and experimental animals may exhibit minor differences in their finer morphologic structure Two or three decades ago—when the rabbit was still the only species in which lesions could be consistently induced—these morphologic details were sometimes heavily emphasized in order to pose the question is this lesion in the rabbit a true counterpart of atherosclerosis in man? Today since atherosclerosis has been successfully induced in virtually every species used in the laboratory this problem no longer looms large The fact is that the stage 1 and 2 lesions in experimental animals are remarkably similar to those in man The dissimilarities are minor considering species differences That is atheroma (the early foam cell plaque) and atherosclerosis (pearly plaques) with additional fresh lipid deposition in the superficial fibrotic layers of plaques—Dr Holman s first two stages—are readily induced in several experimental animals The problem in experimental atherosclerosis has been the relative inability to date to go beyond those two stages i e to effect complications—stages 3 and 4 It is a major problem In the last couple of years several laboratories have begun to make progress toward a solution of that problem Dr Pick will discuss such work in our laboratory We have had some success in producing hemorrhage into plaques ulceration of plaques dissecting aneurysm and on occasion thrombosis superimposed on plaques Dr W S Hartroft in St Louis has—in rats fed thiouracil plus saturated fat plus cholesterol plus cholic acid—apparently produced coronary thrombosis and myocardial infarction (The vessels exhibited only lipid infiltration without plaque formation This is in contrast to the morphologic picture in man wherein coronary thromboses with myocardial *infarctions almost invariably occur only at sites of advanced atherosclerosis*) Thus it would seem valid to suggest that laboratory research is on the way to reproducing the whole gamut of atherosclerotic lesions all four stages in experimental animals But it has not quite succeeded as yet A lot of additional work remains to be done

VOICE Dr Stare and his group have produced far advanced lesions in rats and in monkeys with all the stages which Dr Stamler has just described

POPJÁK I would like to add one or two words about the production of experimental atheroma In those days when I was a pathologist some years ago I studied experimental atheroma and in fact the interest of these years is responsible for my deviation into biochemistry I think it is possible that one of the reasons why we do not *see or have not seen thrombosis in experimental atheroma* is that we have been looking at those arteries in experimental animals in which in the human the lesion is prevalent Now the anatomical features of the coronary

CHAPTER 15

Adrenocortical Hormones and Experimental Atherosclerosis[1]

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The role of the adrenal cortex in lipid metabolism has been suggested by clinical observations in Cushing s syndrome and in Addison s disease The important position of the adrenal cortex in the biosynthesis of steroid hormones led Deuel to the assumption "that it would exert some control over the cholesterol content of the blood

Older studies failed to show changes in serum cholesterol after experimental adrenalectomy This is probably explained by the short survival time of these animals and perhaps by considerable loss of weight water and electrolytes Recent studies performed under adequate experimental conditions revealed a marked decrease in plasma phospholipid and cholesterol concentration in bilaterally adrenalectomized dogs maintained on deoxycorticosterone acetate (DCA) from 8 to 33 days (5 6 11) The main decrease of plasma phospholipid amounted to 50% and that of total cholesterol to 48% of the initial value The substitution of cortisone for DCA resulted in a marked increase in the concentration of both plasma cholesterol and phospholipid whereas discontinuation of cortisone and resumption of DCA therapy again caused marked lowering of both plasma lipid fractions On the basis of these observations and of similar findings in patients treated with adrenocorticotropic hormone (ACTH) and cortisone in whom a striking parallelism between changes in serum cholesterol and phospholipid was observed (3) it was thought that these hormones control the metabolism of plasma cholesterol and phospholipid by the same mechanism

Since the advent of the cortisones considerable information has accumulated regarding the effect of administration of these hormones on circulating lipids and lipoproteins The observations in healthy experimental animals are more valid in this respect than those obtained in human patients with a variety of pathological conditions in whom these hormones were used for therapeutic purposes Observations in normal man are naturally limited by the quantity of the hormones that can be given and by the short duration of the administration Serious adverse effects such as altered cardiovascular dynamics water and salt retention gastrointestinal ulceration and perforation and thromboembolic phenomena represent a well recognized hazard and limit the use of these hormones for experimental studies in man

[1] This research was supported in part by grants from the Division of Research Grants United States Public Health Service

prednisone administration Elevation of the free plasma cholesterol level without a corresponding increase in the esterified fraction was seen in all groups of animals but was more pronounced during prednisone administration These observations may indicate a metabolic block in the esterification of cholesterol as part of the effect of these steroids on lipid metabolism The marked increase in the level of plasma neutral fats may be related similarly to impaired esterification of fatty acids

TABLE II
EFFECT OF PREDNISONE, CORTISONE, AND HYDROCORTISONE ON PLASMA LIPOPROTEIN PATTERNS[a] IN THE RABBIT (10)

Corticosteroids	Animals (no)	Days of treatment (average)	Lipoproteins (% of total stainable lipids) Alpha	Beta	0
None	16	0	30 7	50 4	18 9
Cortisone	2	14	10.2	30 8	59 0
Hydrocortisone	2	29	11.8	33 9	54.3
Prednisone	5	30	5 8	34.5	62 7

[a] By paper electrophoresis

The combination of cortisone (and hydrocortisone) administration with cholesterol feeding resulted in the rabbit, in enhanced elevations of all lipid fractions (Table III) The plasma of these animals showed much greater turbidity than that of the other groups treated with cortisone (or hydrocortisone) alone or with cholesterol supplements alone Despite extreme elevation of all lipid fractions the cholesterol fed hormone injected animals exhibited less atherosclerosis than those treated with cholesterol alone Hormonally induced diminished permeability of the tissue was suggested to explain the experimental findings (4 9) Animals given hyaluronidase exhibited more extensive atheromatous lesions than animals given cortisone which exhibited the least pronounced changes One may reason that hyaluronidase enhanced atheroma formation by increasing permeability of the connective tissue this mechanism was counteracted by cortisone It appears then that factors affecting the ground substance of the arterial wall have an important role in the deposition of lipids

During cortisone therapy in man especially when it was of prolonged duration an elevation of serum cholesterol and phospholipid was not infrequently observed (2) The clinical conditions for which this form of therapy was used included severe systemic diseases such as disseminated lupus erythematosus scleroderma rheumatoid arthritis leukemia, and acute rheumatic fever

To summarize the role of the adrenal cortex in regulating serum

In rabbits the administration of the cortisones produced an increase in plasma cholesterol phospholipid and especially triglycerides (1 7 8) In many instances marked turbidity of the serum (lipemia) was observed Similar changes in serum cholesterol and phospholipid were observed in the rat In contrast in the dog cortisone and hydrocortisone produced only small increases in the plasma lipid fractions

Comparative studies were made of the effects of prednisone cortisone and hydrocortisone on plasma lipids and lipoproteins in the rabbit (10) The increase in phospholipids and neutral fats occurred earlier and was more pronounced than that of cholesterol resulting in moderate changes of the cholesterol phospholipid ratio The increase in serum cholesterol was due to a greater extent to a rise in the free rather than in the esterified fraction The esterified cholesterol fraction decreased from 70–75% of the total cholesterol to 35–60% after 30 days The decrease of esterified cholesterol was most pronounced following prednisone administration Similarly the elevations of all lipid fractions were decidedly higher after 20 and 30 days administration of prednisone than after cortisone and hydrocortisone administration (Table I) There was a marked alteration in plasma lipoprotein patterns (Table II) characterized by a decrease of the alpha and beta lipoproteins and a concomitant increase of the *O* fraction (lipoprotein adhering to the point of origin) Again these changes were most pronounced after

TABLE I

EFFECT OF PREDNISONE CORTISONE AND HYDROCORTISONE ON THE PLASMA LIPID PARTITIONS OF THE RABBIT (10)[a]

		0				10 Days			
Corticosteroid	Animals (no)	Cholesterol (total/esterified)	Phospholipid	Total lipids	Neutral fat	Cholesterol (total/esterified)	Phospholipid	Total lipids	Neutral fat
Cortisone	12	55/41	112	306	139	59/37	154	655	442
Hydrocortisone	11	47/35	103	505	355	42/25	127	771	512
Prednisone	12	50/35	105	350	195	56/33	178	701	467

[a] All values are expressed in mg /100 ml

TABLE I (*continued*)

		20 Days				30 Days			
Cortisone	12	69/29	212	1 090	809	75/45	181	822	566
Hydrocortisone	11	49/25	151	745	545	79/41	250	1 014	765
Prednisone	12	86/40	238	1,260	936	130/46	323	2 056	1 603

[a] All values are expressed in mg /100 ml

lipids and lipoproteins is strongly suggested by recent experimental work in the bilaterally adrenalectomized animal and by the results of hormone administration to normal animals The latter vary with the animal species The role of these hormones in man especially in healthy man is difficult to evaluate and requires additional studies

REFERENCES

1 Adlersberg D Drachman S R and Schaefer L E *Circulation* 4 475 (1951)
2 Adlersberg D Schaefer L E and Drachman S R *J Am Med Assoc* 144 909 (1950)
3 Adlersberg D Schaefer L E and Dritch R *Proc Soc Exptl Biol Med* 74 877 (1950)
4a Adlersberg D Schaefer L E and Wang C I *Science* 120 319 (1954)
4b Wang C I Schaefer L E and Adlersberg D *Circulation Research* 3 293 (1955)
5 DiLuzio N R Shore M L and Zilversmit D B *Federation Proc* 12 197 (1953)
6 DiLuzio N R Shore M L and Zilversmit D B *Metabolism Clin and Exptl* 3 424 (1954)
7 Kobernick S D and More R H *Proc Soc Exptl Biol Med* 74 602 (1950)
8 Rich A R Cochran T H and McGoon D C *Bull Johns Hopkins Hosp* 88 101 (1951)
9 Seifter J Baeder D H Beckfield W J Sharma G P and Ehrich W E *Proc Soc Exptl Biol Med* 83 468 (1953)
10 Wang C I Bossak E T and Adlersberg D *J Clin Endocrinol* 15 1308 (1955)
11 Zilversmit D B Stern T N and Overman R R *Am J Physiol* 164 31 (1951)

DISCUSSION

WHITE The failure of the cortical steroids to intensify the experimental atherosclerosis is of interest in view of clinical experience indicating that these steroids augment lipid mobilization and in view of our own experience in mice in which these hormones appear to exaggerate lipid deposition in the larger blood vessel walls Have you had any evidence of a relationship between adrenal steroid administration and the amount of cholesterol in the diet in other words what does prolonged administration of hydrocortisone do to your normal rabbits and to rabbits who are ingesting varying amounts of cholesterol?

ADLERSBERG We feed rabbits 1 g of cholesterol per day without any additional fat We have no experience with smaller doses of cholesterol Control groups were given various hormones and were fed Purina chow without cholesterol

WHITE Can atheromatous lesions be induced in rabbits receiving hydro cortisone over prolonged periods of time?

ADLERSBERG This is an interesting question We saw only one atheromatous plaque in one rabbit which received hydrocortisone for 4 months Whether this means anything or not I couldn t say Otherwise we saw no atheroma formation in animals treated with corticosteroids without chole terol supplements

STAMLER First with respect to Dr White s last question in chicks as in

TABLE III

PLASMA LIPID FRACTIONS MG PER 100 ML OF THE RABBIT UNDER VARIOUS EXPERIMENTAL REGIMENS 1 GM CHOLESTEROL PER ANIMAL PER DAY (4)[a]

		After 4 weeks				After 2 weeks			After 4 weeks			Degree of atherosclerosis	
Group	Animals (no)	Cholesterol total/esterified	Phospholipid	Total lipids	Additional treatment	Cholesterol total/esterified	Phospholipid	Total lipids	Cholesterol total/esterified	Phospholipid	Total lipids	Aorta	Pulmonary artery
A	9	1006/723	464	2362	None	1223/923	493	3030	1380/1060	486	3270	1 2	0 9
B	5	1098/822	398	2380	Cortisone 3 75 mg daily	2232/1685	785	5040	3501/2392	1031	6969	0 5	0 4
C	13	1013/744	469	2171	Hyaluronidase 1000 TRU and cortisone 3 75 mg daily	1855/1391	658	4490	2697/2004	1098	5613	1 5	0 6
D	14	1105/800	478	2543	Hyaluronidase 1000 TRU daily	1355/1000	571	3363	1376/1040	625	3258	2 0	1 8

[a] Plasma lipid values in normal rabbits in this laboratory are Cholesterol total/esterified—50/35 mg per 100 ml Phospholipids—105 mg per 100 ml Total lipids—350 mg per 100 ml The degree of atherosclerosis is expressed as defined elsewhere (4)

mendously When one studies these animals with P^{3} one sees that the phospholipid synthesis is normal in all tissues but that phospholipids no longer come out of the liver at the normal rate In other words in this preparation the permeability of some membrane in the liver cells to the phospholipid is decreased When one gives these animals cortisone the exchange of phospholipids between plasma and liver increases again so that from this experiment one might conclude that cortisone increases the facility of transfer of phosphatides between the liver cells and the plasma In addition I would like to ask one question about your rabbits We have noticed that our rabbits on high cholesterol diets exhibit severely cholesterol fatty livers jaundice and anemia I should like to ask you when you give these corticoids to your animals does that affect the occurrence of any of these disturbances?

ADLERSBERG In regard to the first question I was very interested to hear that Dr Zilversmit on the basis of his work also is inclined to attribute some role to changes in permeability I may take this opportunity to say that the best evidence so far regarding the role of the adrenals in regulating circulating lipids is that which Dr Zilversmit and Dr DiLuzio in his laboratory presented that is that adrenalectomized dogs maintained on DCA show regularly a drop of approximately 50% in serum cholesterol and phospholipid If DCA is replaced by cortisone the levels return to normal This is probably the best evidence that cortisone plays a role in regulating levels of serum lipids These animals developed high blood sugars We were not sure that the diabetes produced by the hormones was responsible for the serum lipid changes Characteristic histologic changes in the pancreas have been described since by workers in this field

HOWARD May I ask a few questions of Dr Adlersberg concerning the first study? Was the 1% cholesterol diet fed *ad libitum* to these animals or was it a constant amount per day? What were the exact daily doses for the cortisone hydrocortisone and prednisone? Finally what were the final weights of the animals compared to the control weights?

ADLERSBERG In regard to the diet and the quantity of cholesterol I should have quoted Dr Popják as the originator of the technique using cholesterol alone without any other fat vehicle for producing hypercholesterolemia in animals We prepared a special chow which contained 1 g of cholesterol per 100 g of Purina rabbit chow The rabbits eat it and thus consume approximately 1 g of cholesterol per day Their serum cholesterol phospholipids and triglycerides rise in the way described

After many preliminary studies the dosage was 3 5 mg of hormone per day regardless of whether it was cortisone hydrocortisone or prednisone per 3 kg rabbit We preferred larger rabbits because it was easier to draw blood from their ear veins The final weight of the animals was often somewhat higher than the original weight because they were still growing And they ate very well if no large doses of the hormones were used which produced toxic effects

PINCUS I would like to interject one thing here which I think is relevant In the rabbit the endogenous corticosteroid is none of these that you use but chiefly corticosterone This has been shown by a number of investigators I imagine you have not used corticosterone unless you have not described it but in the ACTH administered animals is there any difference particularly in the nature of the lesions as compared with the animals receiving the other corticosteroids?

ADLERSBERG In the studies with ACTH we did not use too many animals The effects on lipids were milder than those with cortisone But again the question

rabbits administration of hydrocortisone without cholesterol feeding led to a marked hypercholesterolemic hyperlipemia However this regressed in a couple of months although the hydrocortisone induced hyperlipemia persisted We don't know why that regression occurred no lesions were observed With hydrocortisone administration and cholesterol feeding in chicks our results were quite similar to those of Dr Adlersberg in rabbits marked enhancement of hyperlipemia no significant intensification of atherogenesis Finally I regret to say that with hyaluronidase we observed no effects in chicks in contrast to Dr Adlersberg's findings in rabbits

ROSENMAN The failure of the steroid enhanced degree of hypercholesterolemia to increase the severity of atherogenesis may perhaps be more reasonably explained by the marked rise of plasma triglyceride induced by the steroid administration A similar lack of enhanced or even diminished atherogenesis also occurs in cholesterol fed rabbits concurrently given Triton or alloxan their plasma again exhibiting a marked rise of triglyceride

In recent studies from our laboratory it was found that cholesterol has a preferential solubility in excess triglyceride and indeed the hypercholesterolemia of Tritonized and of nephrotic rats was shown to be a passive accumulation of cholesterol secondary to the lipid sequestering capability of the increment of excess plasma triglyceride respectively induced by the effect of Triton or by the deficiency of albumin in nephrotic plasma A similar sequence was found by Dr Friedman and Dr Byers to occur in normal rats in which a proportionate rise of plasma cholesterol was shown to occur when normal rats were continuously infused with various triglycerides The ability of the excess triglyceride increment to hold cholesterol intravascularly was further indicated by the fact that in each of these experimental situations the accumulation of excess cholesterol was confined to the plasma

Thus the trapping of excess cholesterol in the plasma as the result of its preferential solubility in the excess triglyceride fraction found in the plasma of the animal concomitantly fed cholesterol and given steroids Triton or alloxan may explain the failure of atherogenesis to be enhanced in such animals despite the augmented hypercholesterolemia A clinical parallel may be exemplified by patients with idiopathic hypercholesterolemia since such patients exhibit severe hypertriglyceridemia as well as marked hypercholesterolemia but do not generally exhibit the very severe atherogenesis frequently occurring at very early ages in patients with essential hypercholesterolemic xanthomatosis whose plasma although hypercholesterolemic is not characterized by a significant increase of triglycerides

ADLERSBERG *To answer the remarks of Dr Stamler much depends on the* species and the doses you use The bird has a completely different lipid metabolism than the mammal and one has to be extremely careful in comparing the tissues and the lipid metabolism in various species

As to the remarks of Dr Rosenman I would not like to imply that diminished or increased tissue permeability is perhaps the only factor responsible for our observations I think it is one of the factors which should be considered in our work in this field I am aware of the work of Drs Byers and Friedman and of their concept that the lipemia has a trapping effect on serum cholesterol There are probably some other possibilities as well which canot be discussed here

ZILVERSMIT I should like to comment on the matter of permeability In adrenalectomized dogs maintained on DCA the serum lipid levels drop tre

Experimental Stress Blood Lipids, and Atherosclerosis

MEYER FRIEDMAN AND HERMAN UHLEY[1]

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A recent review by Wilhelm Raab (4) points out that much evidence is available concerning the atherogenic effectiveness of epinephrine and norepinephrine both in animals and man that this effectiveness is enhanced by thyroid hormone and that, in general emotional sympathetic overstimulation may be assumed to be damaging to the arterial walls A L Myasnikov (3) has summarized much Russian work on the influence of central nervous factors and neurotropic drugs on cholesterol induced atherogenesis in rabbits Phenobarbital markedly reduced the atheroma of the rabbits without changing blood lipid values Amytal and chloral hydrate had similar though less marked, effects Benzedrine increased both blood lipids and atherosclerosis although caffeine did not

Observations on 128 patients treated with the same drugs led the Russian authors to conclude that the cholesterol concentration of blood is controlled by the central nervous system Wexler and Miller (7) were able to produce what they describe as a fulminating arteriosclerosis in the old female rat solely by administration of adrenocorticotropic hormone several times a week for 7 weeks while these animals were fed an ordinary laboratory diet without added fat

All these studies indicated the importance of stress in the genesis of atherosclerosis

The two experimental situations to be described herein were devised in an attempt to approximate in animals some of the stresses of human group living The authors do not believe that a very close approximation was achieved but feel that the philosophy of this attempt is valid and may lead ultimately to quantitation of the somatic effects of psychic factors in human stress

In the first experiment (6) a selection of 30 most "aggressive" and 30 most "passive" individuals was made from a commercial flock of 2500 male chickens by an experienced handler The groups were separately housed and fed twice daily only the amount of food which would be consumed within 1 hour by the passive group The diet was mash enriched with 5% cotton seed oil and 2% cholesterol Observation of the behavior of the fowl was continued for the 15 weeks of the experiment and a further selection made of a total of 12 aggressive and 9 passive chickens which had exhibited a consistent behavior through

[1] Presented by Sanford O Byers

of dosage comes into the picture It is quite possible that if we had used larger quantities of ACTH the results would have been different

FURMAN In connection with Dr Zilversmit s remarks regarding the status of the liver it is interesting to observe that the elevation of cholesterol observed by Dr Adlersberg was mainly in the "free or unesterified fraction Hypercholesterolemia due mainly to elevation in the unesterified fraction is seen in biliary obstruction Failure for normal amounts of esterified cholesterol to obtain can also be interpreted as evidence of hepatic insufficiency I wonder if Dr Adlersberg would speak more directly to Dr Zilversmit s question regarding histologic or functional evidence of liver derangement because some of the changes in circulating lipids which he described could be secondary to liver damage and not a primary effect of the steroid administered to the animal

ADLERSBERG I think this is a very good possibility *Dr Furman* except that on histologic examination of these livers we did not find any evidence of hepatotoxic effects

to form a visible "endogenous chylomicronemia" and finally (5) simultaneous accumulation of plasma phospholipid and cholesterol in excess These events except for the initial kidney damage can all be shown to be reversed by intravenous administration of bovine albumin to the rat

The sequence after administration of Triton WR 1339 a wetting agent (2) appears to be (1) accumulation of plasma triglyceride (triglyceride does not appear in the hepatectomized animal however if triglyceride is supplied intravenously the sequence follows even in the liverless animal) to the point of endogenous chylomicronemia" followed by (2) simultaneous appearance of excess plasmaphospholipid and cholesterol

The sequence after bile duct ligation (1) appears to be (1) accumulation of bile acid then (2) accumulation of plasma phospholipid and finally (3) appearance of excess plasma cholesterol. The hypercholesteremic plasma is clear and transparent in the bile duct ligated rat

Each of these sequences may be initiated at any stage by intravenous injection and *continuous maintenance* in the plasma of substantially elevated concentrations of the appropriate blood lipid

References

1 Friedman M and Byers S O *Am J Physiol* **188** 337 (1957)

2 Friedman M and Byers S O *Am J Physiol* **190** 439 (1957)

3 Myasnikov A L *Circulation* **17** 99 (1958)

4 Raab W *Am J Cardiol* **1** 113 (1958)

5 Rosenman R H Byers S O and Friedman M *J Clin Invest* **36** 1558 (1957)

6 Uhley H Friedman M and Ayello C *Proc Soc Exptl Biol Med* **96** 244 (1957)

7 Wexler B C and Miller B F *Science* **127** 590 (1958)

Discussion

EDER An alternate explanation to the hypothesis suggested by Dr Byers to explain the changes in nephrosis is that suggested by the observations of Dr David Gitlin He showed that in nephrotics there is a decreased transformation of the very low density beta lipoproteins to higher density beta lipoproteins These low density beta lipoproteins contain appreciable amounts of triglyceride as well as smaller amounts of cholesterol and phosphatide The failure of this conversion of the very low density beta lipoproteins to ones of higher density may be explained in terms of the lipoprotein lipase (clearing factor) This enzyme hydrolyzes the triglyceride of the low density lipoproteins and conceivably converts them into ones of higher density In the nephrotic this decrease in activity of the enzyme may be a reflection of a decreased concentration of serum albumin which binds the fatty acids released on the hydrolysis of triglyceride These fatty acids may accumulate and inhibit the enzyme

out Body and organ weights of the two groups were similar except for heavier testes and combs in the aggressive group At autopsy the degree of coronary atherosclerosis was evaluated as identical in both groups Average aorta cholesterol was 1264 mg/100 g in the aggressive group and 1957 mg/100 g in the passive group The average plasma cholesterol during the experiment was 348 mg % in the aggressive group and 525 in the passive group The results of this experiment ran counter to the expectation of the authors who had anticipated a positive correlation of aggressiveness with atherosclerosis

The second experiment was designed as an atttempt to simulate in rats the state of mind of a busy executive with more appointments than he has time for or of a high pressure salesman driven to beat his own past sales record Rats were placed for 6 hours daily in a cage with provisions for electrically charging the floor with a faradic current adjusted to administer a harmless shock Each half of the cage floor was charged alternately at random intervals The animals soon learned to seek the uncharged area and remain there until it became charged whereupon they shifted position However since they could not predict when such a change would be necessary they were constantly alert and poised for quick action This alertness is clearly shown in the accompanying motion picture The first such experiment is in its fifth month at the present time No autopsies have been performed as yet The 15 experimental animals now average 255 g in weight the controls 270 g Blood cholesterol and phospholipid values have similar averages in both groups Both are high because these animals are eating an atherogenic diet The most striking difference between the two groups is in blood clotting time as determined in capillary glass tubes The blood clotting time of the experimental group averages 64 seconds with a range of 40 to 90 seconds that of the control group averages 111 seconds with a range of 60 180 seconds

This shortening of blood clotting time gains significance when considered in relation to the possibility of excessive occurrence of thrombotic accidents in individuals under time stress This latter possibility will be dealt with at length by Dr Ray Rosenman in his presentation this evening

Dr Rosenman has also asked me to present some of the work we have done on the sequence of events in various experimental hypercholesteremic states as he will not have time to do so tonight Briefly the sequence in experimental nephrosis in the rat appears to be as follows (5) (*1*) kidney damage (*2*) loss of plasma albumin to a concentration of less than 1 g % (*3*) failure of lipoprotein lipase "clearing" mechanism (*4*) accumulation of excess plasma triglyceride sufficient

tions One as I watched the behavior of the members of this conference I wondered whether the San Francisco group would not prefer to add the busy scientist to the business man and the salesman Two was the design of the experiment at all colored by the fact that we talk about life being a rat race?

Now to be serious What about the ability of these animals to eat as compared to control animals subject to electrical shock? What about exercise itself as distinct from exercise and suspense? There is some recent work which suggests that animals who exercise have less atherosclerosis and less hypercholesterolemia Now how have these two variables been controlled in the design of the experiment?

BYERS In reply to the second of Dr Katz facetious remarks the design of this experiment was due most largely to the fact that Dr Herman Uhley was trained in electronics in the Radar Corps during the last war and the particular electric circuit and the notion of the charged floor and so on are influenced by his ideas of what would move a rat without damaging it

The food that the two groups eat is comparable They have not been strictly pair fed but the food that they eat has been measured and until recently I believe the ingestion of food was comparable However I understand from Dr Friedman that steps are now being taken to limit both groups to the amount of food taken by the group which eats the least With regard to exercise it seems obvious to me that the nonstimulated rats have a more congenial type of exercise than the *stimulated ones When one considers that the rat normally sleeps in the daytime* or at least is a nocturnal animal then these rats are really in a rat race They are going night and day

WALKER I would like to add to what Dr Katz has mentioned in relation to activity and its role in atherosclerosis and coronary heart disease Most of you will be aware of the studies of Dr Morris in England dealing with coronary death rates in populations with different levels of physical activity In a paper given at the Washington Conference on "Cardiovascular Epidemiology Morris noted that postmen have a lower death rate from coronary heart disease than less active post office clerks Similarly he showed that bus conductors have a lower coronary disease mortality than bus drivers Furthermore there is the generally more favorable position of rural (and presumably more active) dwellers as against those living in urban areas Biochemically there is a certain amount of information bearing on this subject There are the studies of Dr George Mann and co-workers concerning the influence of muscular exercise in lowering serum lipid levels There is evidence that fibrinolysin activity in the blood is stimulated by physical exercise Furthermore in a paper just published by Dr Connell and co-workers it was shown that physical training can have a highly significant lowering effect on the excretion of 17 ketosteroids One presumes that other aspects of the endocrinological picture are also affected under these conditions Therefore I think that in both experimental work and in studies on humans there is room for a considerable amount of further investigation in relation to the role of activity

STRISOWER I would like to ask whether the triglyceride that was used was prepared as an emulsion

BYERS We have used three or four different emulsions We have used Lipomul and controlled it with Lipomul vehicle we have used Baxter s fat prepared for intravenous alimentation We have used coconut oil almond oil olive oil and other types It is all animal work and these emulsions have not been handled aseptically

BYERS We have not postulated any greater than normal withdrawal of cholesterol from body depots as necessary to explain the hypercholesteremia of experimental nephrosis We believe the rate of rise of plasma cholesterol in this condition to reflect the normal rate of entry of tissue (liver for example) cholesterol into the blood The rate of withdrawal of blood cholesterol for further metabolism by tissue is slowed down in nephrosis The blood cholesterol level merely reflects the disparity between a normal rate of income and a slowed rate of outgo

With regard to the hypotheses of Dr Gitlin I should like to say that we believe *all* the syndrome following experimental nephrosis is explicable solely on the basis of loss of plasma albumin We can reverse the syndrome by administration of albumin alone or we can prevent onset of the syndrome in the face of typical kidney pathology by preventing loss of urine containing albumin We can initiate the sequence of plasma lipid changes *in vivo* at any stage by administration of the proper lipid at a rate such as to mimic the rate of its accumulation in the experimental syndrome Of course the lipids we inject are not in physiological lipoprotein form and therefore strictly speaking do not exactly duplicate the natural state However the experimental analytical values for each plasma lipid are exactly duplicated following appropriate injection of a lipid early in the sequence triglyceride phospholipid cholesterol

We can demonstrate *in vitro* precisely the defect in lipoprotein lipase action postulated *in vivo* and we can remedy this defect *in vitro* solely by addition of albumin to the nephrotic plasma in the test tube We can inject albumin into the nephrotic rat withdraw some and show that the lipoprotein lipase defect is abolished simultaneously with alteration of plasma lipids in the direction of normality

BOYLE This failure of clearing in these two situations I think is entirely different in Triton rats and the nephrotic rats In the incident of the Tritonized rat the failure to clear is due to a specific inhibition of the substrate In other words these animals can make clearing factor and if their lipoproteins are separated by centrifugation the addition of their heparinized plasma will be able to clear normal chylomicrons Whereas the Triton treated rats chylomicrons cannot be cleared in a normal clearing factor system so that the blockage in the presence of Triton apparently is the blockage of the enzyme site on the substrate chylomicrons In the incident of nephrosis I think the hypoalbuminemia plays a major role whether it is all quantitative or qualitative we dont know But in one case of idiopathic hypoalbuminemia that Dr Robert Gordon was studying at the Heart Institute (I helped in the laboratory work with him on this) when this idiopathic albuminemic patient s albumin became less than 1 g % she became hyperlipemic and hypercholesterolemic in the range of 300 to 600 mg % cholesterol elevated in the low density lipoproteins If you administered one unit of albumin her lipid values promptly fell to normal to about 160 to 180 mg % cholesterol She had no renal disease detectable by any known means This shows that perhaps the hyperlipemia and the hypercholesterolemia in nephrosis are not specific to renal disease but secondary to either qualitative or quantitative alterations in albumin blood levels The clearing mechanism fails to work in these two conditions by different mechanisms one with the acceptor protein for free fatty acids being inadequate and in the other incidence the substrate being blocked by the wetting agent Triton

KATZ I want to make two facetious remarks and then ask some serious ques

associate Dr Meyer Friedman Thus Dr Friedman noted that Dr Morris and his associates failed to contrast in their own data the varying incidence of coronary disease in the downtown (central) compared to the suburban transport personnel Having learned that both the downtown bus drivers and conductors in London are constantly exposed to a severe form of occupational stress largely absent in the suburban personnel I believe that Dr Friedman's observations are particularly significant Thus as can be noted in the recalculated data of Dr Morris which are presented in Table A the incidence of angina and myocardial infarction and the total incidence of clinical coronary disease are each greater in the downtown drivers compared to the suburban drivers and are similarly greater in the downtown conductors than in the suburban conductors Of particular significance is the fact that the *downtown conductors* have a higher total incidence of coronary disease than that exhibited by the *suburban bus drivers*

I would also like to comment on the reference to Dr Gitlin's studies in nephrotic patients which was made by Dr Eder earlier To say that hypoalbuminemia is present in nephrotic plasma is to characterize descriptively a finding which of course does not explain the mechanism of its occurrence In analogous fashion I believe it is a semantic error to state that the hypercholesteremia of nephrotic plasma or in other hypercholesteremic states is *due* to hyperbetalipoproteinemia The latter is again a descriptive characterization which fails to explain either its own occurrence or the occurrence of hypercholesteremia It would rather appear to me merely to describe the manner in which the excess increment of cholesterol is transported in the plasma Dr Gitlin has found that the excess cholesterol in nephrotic plasma is transported as low density beta lipoproteins having a slower than normal rate of turnover I do not believe that this finding is at all at variance with our earlier findings that nephrotic hypercholesteremia is ascribable to the markedly delayed plasma egress of cholesterol a consequence of the associated hypertriglyceridemia in turn ascribable primarily to the deficiency of circulating albumin in nephrotic plasma

STRISOWER There exists some evidence that fat intake is a stimulus for the appearance and/or increase of clearing factor in human and rat plasma Injection of a triglyceride emulsion however may evoke different mechanisms since one is dealing here with an unphysiological situation due to differences in physical and chemical properties of triglyceride emulsions and S_f^0 20–400 serum lipoproteins and chylomicra

ADLERSBERG Dr Patterson a well known pathologist in Canada published a short paper or only an abstract of a paper several years ago in which he put chicks under stress somewhat similar to the one that you use His observations were that *these chicks kept always in a status of fright and motion presented* less atherosclerosis than controls fed cholesterol only I would like to say that we have been interested in the effects of intravenous fat infusions in man A single dose consists of 600 cc and supplies 90 g of neutral fat as purified corn oil This quantity is usually given intravenously within 3 hours One finds practically no changes from what we have seen in the serum cholesterol and phospholipid levels Of course the triglycerides do go up and the serum may become turbid Clearin after a few hours is seen If these infusions are repeated regularly the clearin mechanism suffers probably by exhaustion of the lipoprotein lipase reserves

ROSENMAN I would like to comment on Dr Walker s reference to the studies of Dr Morris and associates concerning the incidence of clinical coronary heart disease in London transport workers (*Lancet* 2 1054 1953) Dr Morris has emphasized the factor of exercise in accounting for the lower incidence of *clinical* coronary heart disease in the London bus conductors compared to the drivers When the data presented by Morris *et al* are recalculated in terms of the incidence of cases per 1000 man years observed as has been done in Table A it can be

TABLE A

COMPARISON OF INCIDENCE OF CLINICAL CORONARY HEART DISEASE IN LONDON TRANSPORT WORKERS[a]

Location of transport personnel	Type of transport personnel	Incidence of coronary disease (number/1000 man years) Angina pectoris	Myocardial infarction	Total incidence
All transport drivers		0 32	2 28	2 60
All transport conductors		0 63	0 99	1 62
Only drivers	Downtown (Central)	0 42	2 56	2 98
	Suburban (Trams)	0 11	1 61	1 72
Only conductors	Downtown	0 77	1 23	2 0
	Suburban	0 32	0 48	0 8
Conductors	Downtown	0 77	1 23	2 0
Drivers	Suburban	0 11	1 61	1 72

[a] Data derived from that presented by Morris *et al*

seen as noted by the authors that the incidence of myocardial infarction and the total incidence of clinical coronary disease but not that of angina are higher in the drivers than in the conductors

However I wonder about the validity of Dr Morris conclusions in view of the most interesting observations concerning his data that have been made by my

Experimental Atherosclerosis in Dogs[1]

JESSIE MARMORSTON SHELDON ROSENFELD[3] AND JOHN MEHL

Institute for Medical Research Cedars of Lebanon Hospital and Department of Medicine and Biochemistry University of Southern California Los Angeles California

To date we have studied over 70 dogs and have produced experimental atherosclerosis either by using a thiouracil and cholesterol regimen or by performing total thyroidectomy followed by a high cholesterol diet Each dog has been individually fed thiouracil and/or cholesterol on the basis of its body weight and all animals have had free access to exercise We have been interested in studying three major points (*a*) the effect of thyroidectomy as compared with thiouracil treatment (*b*) the role of stress in the production of the disease and (*c*) the relationship between the development of the disease and the urinary excretion levels of corticoids and estrogens It has been suggested that there is a breed difference in the susceptibility of the dog to the development of experimental atherosclerosis Dr Kendall has been inbreeding a strain of dog which he believes is more susceptible to the development of the disease In studies conducted on some of these normal dogs ("Kendall mongrels) and normal beagles it was observed that in the Kendall mongrel the serum cholesterol and phospholipid levels were significantly higher The average serum lipid values for the beagle were cholesterol 158 mg % phospholipid 329 mg % cholesterol phospholipid ratio (C/P) 0 48 and beta lipoprotein 28% The average serum lipid values for the Kendall mongrel were cholesterol 218 mg % phospholipid 382 mg % C/P ratio 0 55 and beta lipoprotein 32%

Eight beagles were put on the thiouracil and cholesterol regimen for a period of 10 to 12 months Figure 1 shows the typical response of a beagle on this regimen The average serum lipid values rose as follows cholesterol from 158 to 512 mg % phospholipids from 329 to 495 mg % C/P ratio from 0 48 to 1 04 and the beta lipoprotein from 28 to 44% Six Kendall mongrels were put on the thiouracil and cholesterol regimen for periods up to 8 months The average serum lipid levels rose as follows cholesterol from 218 to 718 mg % phospholipid

[1] The investigations described in this paper were aided by grants from the Lasker Foundation and the United States Public Health Service (H 3381)

Introduction by Jessie Marmorston M D "Dr Sobel collaborated with me in the planning of the experiments herein reported His critical appraisal of the work particularly in its early phases is gratefully acknowledged

[3] Presented paper

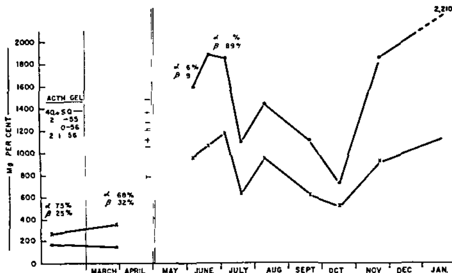


Fig 3 Effect on serum lipid values of ACTH given 4 months prior to the administration of thiouracil and cholesterol (beagle dog No 19 ♂)
Key ● = cholesterol x = phospholipid

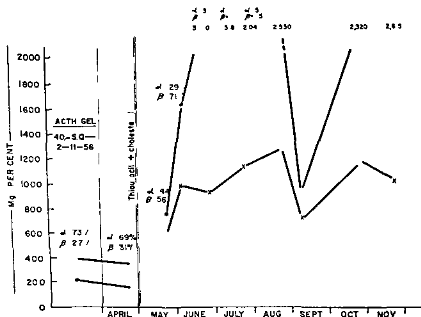


Fig 4 Serum lipid response of ACTH pretreated Kendall mongrel to thiouracil and cholesterol regimen (dog No 47 ♂)
Key ● = cholesterol x = phospholipid

from 382 to 588 mg % C/P ratio from 0 55 to 1 22 and beta lipo protein from 32 to 48%

When thyroidectomy replaced thiouracil the average serum lipid response was greater in the male than in the female dog Figure 2

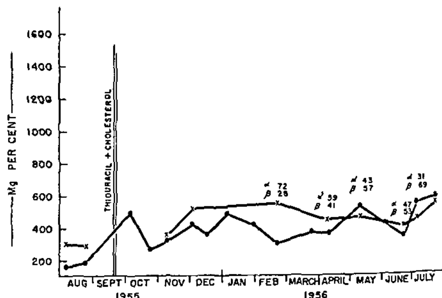


Fig 1 Typical response of a beagle on thiouracil and cholesterol regimen (beagle dog No 7 ♂)
Key ● = cholesterol x = phospholipid

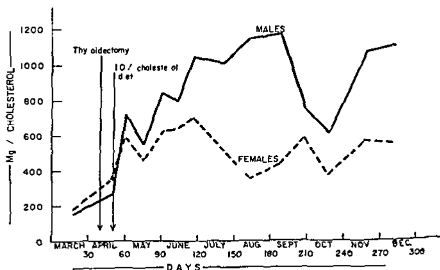


Fig 2 Average values for 11 beagles (6 females 5 males) with thyroidectomy and cholesterol

lesterol treatment In the dogs given ACTH 2 to 4 months prior to the administration of thiouracil and cholesterol there were no changes in the serum lipid values until after thiouracil and cholesterol was added to the diet Eleven dogs were studied in this group Figure 3 illustrates this effect In the beagle the serum lipid elevations were as follows cholesterol went from 158 to 350 mg % phospholipids went from 329 to 830 mg % C/P ratio went from 0 48 to 1 67 and the beta lipoprotein

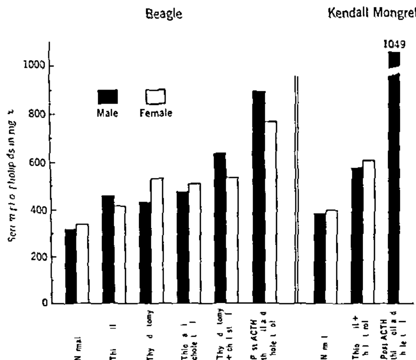


FIG 6 Average serum phospholipid response of beagle and Kendall mongrel to the various experimental procedures

went from 28 to 75% The serum lipid response obtained from the Kendall mongrel (Fig 4) was as follows cholesterol went from 218 to 2530 mg % phospholipids went from 382 to 1049 mg % C/P ratio went from 0 55 to 2 41 and the beta lipoproteins went from 32 to 83% In addition of the 11 animals treated with ACTH 4 have experienced some form of paralytic stroke

Figures 5 6 7 and 8 summarize the lipid response of the beagle and Kendall mongrel to the various experimental procedures The most important point to be made at this time is that under the conditions of

demonstrates this effect of thyroidectomy on 11 animals 6 females and 5 males Though the data to date are highly suggestive of a male female difference a greater number of animals is needed for statistical significance This study is presently being pursued

In order to investigate the role of stress in the development of atherosclerosis we have used large single or multiple doses of ACTH (adreno

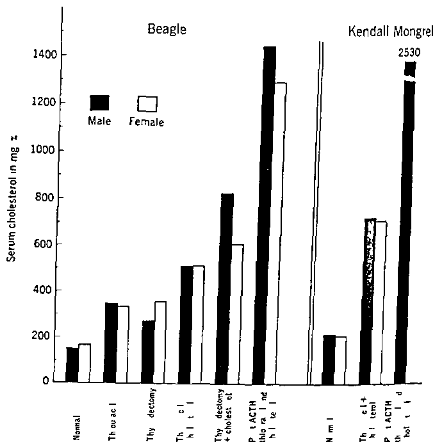


FIG 5 Average serum cholesterol response of beagle and Kendall mongrel to the various experimental procedures

corticotropic hormone) electric shock and cold stress We found that when beagles Kendall mongrels or mongrels were treated with 1 to 3 subcutaneous injections of 40 units of ACTH there was a marked increase in the serum lipid response of these animals to the subsequent feeding of a standard thiouracil and cholesterol diet Furthermore it made no difference whether the animals received the ACTH 2 to 4 months prior to or at the commencement of the thiouracil and cho

tomy and before the animal is fed cholesterol and becomes hyper cholesterolemic there is an alteration in the urinary excretion of es trogens This effect is illustrated in Fig 9 The normal level of urinary estrogen excretion for 25 determinations on 17 male beagles is 0 033 µg per 24 hours for 21 determinations on 16 female beagles it is 0 047 µg per 24 hours The preliminary "sensitizing procedures" cause the urinary estrogen excretion to fall from 0 033 to 0 015 µg per 24 hours in the male and from 0 047 to 0 026 µg per 24 hours in the female dog

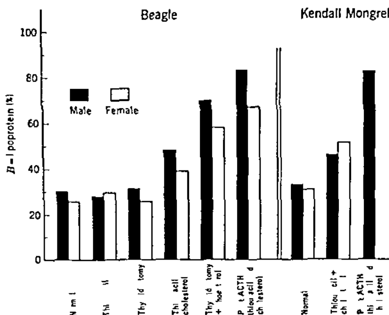


FIG 8 Average serum β-lipoproteins of the beagle and Kendall mongrel after various experimental procedures

After cholesterol feeding and the development of hypercholesterolemia the urinary estrogens average 0 051 µg per 24 hours in the male and 0 043 µg per 24 hours in the female It should be noted that whereas the urinary estrogens return to the normal level in the female in the male the urinary estrogens exceed the normal level Furthermore in 34 determinations on male dogs and 33 determinations on female dogs a positive correlation exists between the serum cholesterol and the urinary estrogen levels This correlation is significant to less than 0 001 over serum cholesterol ranges up to 5000 mg %

Figures 10 11 and 12 illustrate the gross pathology Figure 10 shows the aorta stained with Sudan IV used to demonstrate the lipid content

this experiment ACTH greatly sensitizes the dog to the atherosclerotic effect of thiouracil and cholesterol The lipid elevations produced in the ACTH plus thiouracil and cholesterol treated group are significantly greater than the lipid elevations produced in the thiouracil and cholesterol treated group ($P = 0.001$) Tests of significance were not made for comparison of the beagle and Kendall mongrel in all experimental

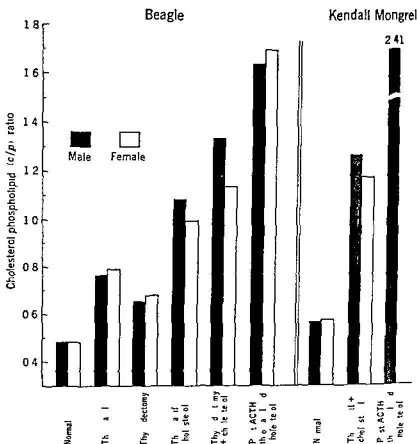


Fig 7 Average cholesterol phospholipid (C/P) ratio of beagle and Kendall mongrel after various experimental procedures

groups because there were not enough Kendall mongrel dogs in all groups However it does appear that the Kendall dog is more susceptible to the development of experimental atherosclerosis

We have been interested in the urinary excretion of estrogens during the development of atherosclerosis At this time we have preliminary data which suggest that during the periods of sensitization during which time thiouracil or ACTH is given or immediately after thyroidec



Fig 10 Aorta stained with Sudan IV demonstrating the lipid content of the atheromatous lesions

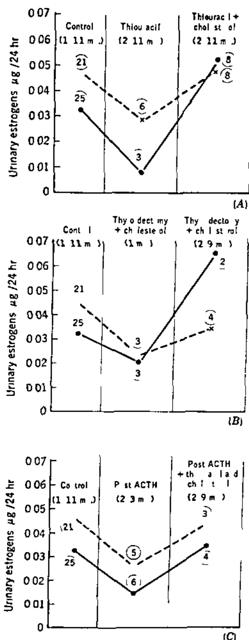


Fig 9 The effects on beagle urinary estrogens (µg /24 hr) of (A) thiouracil (2–11 months) and thiouracil plus cholesterol (2–11 months) (B) thyroidectomy and cholesterol (1 and 2–11 months) and (C) post ACTH (2–3 months) and post ACTH plus thiouracil plus cholesterol (2–9 months)

Key ●——● male (♂) x x female (♀) # number of determinations

the stroke the animal was completely paralyzed, gradually motor function returned to the head and progressively to the posterior extremities It has been our policy to withdraw thiouracil and cholesterol at the



Fig 12 Brain of dog who died very suddenly from a cerebral hemorrhage in the region of the posterior part of the circle of Willis

time of the stroke to help recovery and not to re administer thiouracil and cholesterol until maximum recovery has occurred. This has been done to simulate the pattern of treatment in man

Other forms of stress have been used in this study Figure 13 shows the effect of electric shock on the urinary excretion of corticoids

of the atheromatous lesions Figure 11 illustrates the marked degree of involvement of the coronary vessels The cerebral arteries and most of the smaller vessels of the body were also severely involved Figure 12



FIG 11 Coronary vessels with marked degree of involvement

shows a brain of a dog who died very suddenly from a cerebral hemorrhage in the region of the posterior part of the circle of Willis A short film shows the residual effects observed in a dog 1 month after suffering from a paralytic stroke This is a dog who was treated with ACTH plus thiouracil and cholesterol for 12 months During the first 2 weeks after

hour and electric shock causes the urinary corticoids to rise to levels of 140 to 160 µg per hour Figure 14 shows the effect of cold stress on the urinary corticoid creatinine ratio In 4 normal dogs cold stress produced no change in the urinary corticoid creatinine ratio whereas in 5 ACTH plus thiouracil and cholesterol treated dogs cold stress caused the corticoid-creatinine ratio to rise from 0 0025 to 0 0046 The relationship of cold and electric shock stress to the ACTH phenomenon the mechanism of the ACTH phenomenon and the effects of estrogen therapy and exercise on experimental atherosclerosis are currently being studied

DISCUSSION

HOLMAN First I would like to compliment you and your co-workers on the production of a cerebral hemorrhage I know of no previous work like this and I would like to inquire a little more about it such as the exact site and nature of the hemorrhage Do you know whether it came from the basilar artery or from one of the major vessels at the base of the brain? Has the hemorrhage in the other dogs been subdural or subarachnoid in nature as suggested by the gross picture which you showed? Have you done spinal taps on any of these dogs?

STAMLER Is that lesion a cerebral hemorrhage or a hemorrhagic infarct?

ROSENFELD Gentlemen I am not a pathologist We are sending these tissues to a pathologist for a complete report In my opinion it appears to be a subarachnoid hemorrhage in the region of the posterior portion of the circle of Willis The specimen is here if any of you are interested in examining it

KENDALL I might say that we have observed this sequence of symptoms that is the apparent partial paralysis and long drawn-out convalescence from the condition when taken off from the thiouracil-cholesterol regimen but we never have been able to produce any evidence of cerebral damage We have had brains from animals dying in this condition examined by a competent neuropathologist and he was unable to locate any areas of hemorrhage nor could he find any changes that would enable him to refer the symptoms to any specific cerebral damage

STAMLER I may be able to throw some light on this hind leg paralysis based on purely personal family experience We have had this syndrome in our cocker spaniel at home and we learned from the veterinarian that this is a very common occurrence in dogs Apparently dogs readily develop damage to the lower spinal cord with resultant hind limb paralysis Now this observation in no way gainsays the validity of the cerebral hemorrhage presented here It is merely to suggest the possibility that in some of the living dogs hind limb paralysis may develop on a quite different basis

OLIVER I wonder if Dr Rosenfeld would perhaps elaborate on the precise features of this so-called paralysis Did this involve all four limbs did it involve one limb was it spastic was it flaccid and what are the chances that this could be an event which did not involve any brain lesion? You did in fact say that the dog would not respond just lay about for a month Now if this were due to a quadriplegia and bilateral cortical hemorrhage one would wonder at the dogs survival Experimentally induced damage in various aspects of the cortex of the dog will produce lesions very comparable to those seen in the human and there

beagles and mongrels In the beagle the normal urinary corticoids as measured by the Silber Porter method are about 120 µg per hour Electric shock *produces little or no effect on the hourly corticoid excretion* In the mongrel the normal urinary corticoid level is about 80 µg per

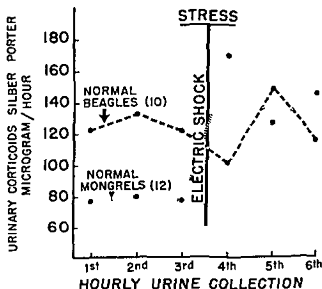


Fig 13 Effect of electric shock on the urinary excretion of corticoids (as measured by the Silber Porter method) in beagles and mongrel

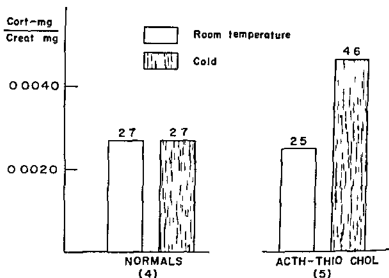


Fig 14 Urinary corticoid creatinine ratio of normal and ACTH thiouracil cholesterol treated dogs before and after cold stress

ceiving ACTH what was the time interval between administration of ACTH and the manifestation of the cerebral symptoms

ROSENFELD Most of the animals were given 1 to 3 injections of ACTH 2 to 4 months prior to the feeding of thiouracil and cholesterol We have given some animals ACTH at the same time that they were given thiouracil and cholesterol and others have been given ACTH after they had been on thiouracil and cholesterol a long time One of the animals given ACTH after being hypercholesterolemic for a long time was one of the animals cited as having this condition and two of the animals who were given the ACTH and thiouracil and cholesterol at the same time developed cerebral occlusive attacks after 8 months The third animal was one of the animal who had received the ACTH prior to the thiouracil and cholesterol

MARMORSTON In regard to Dr Oliver s question which I believe was not clearly answered in all instances paralysis preceded the cerebral lesion I think this is not an accidental finding As to the exact location of the cerebral lesion and its relationship to the neurological manifestations in the particular dogs we are not able to answer that at this time because we have not studied the pathology of these lesions microscopically as yet We have about 35 dog brains quite ready to section and perhaps next time we shall be able to give a more precise answer I think this also covers Dr Stamler s question as to whether this is possibly an accidental paralysis

fore it might be of some interest to know the precise neurological and pathological findings in these animals

ROSENFELD In answer to Dr Stamler I am familiar with the problems of household pets as I am a veterinarian as well as a physiologist This is not the condition you describe In this particular animal one side was involved and the animal moved around in circles In another case the animal was completely paralyzed and could not eat or swallow for 5 days Eventually there was an increase in motor activity which was progressive from the head down the animal began to use his tongue could eat and eventually was able to regain his stability

HELLMAN Do you have any information about the blood pressure of these dogs?

ROSENFELD No I dont The only excuse we have for not doing this was that after seeing these lesions I was worried about puncturing the femoral vessels We are planning to study blood pressure and peripheral blood flow in these animals

STRISOWER Dr Rosenfelds comments are of great interest to me particularly with respect to observations made by Dr Wei Young in humans concerning the correlation between coronary and cerebral atherosclerosis These were autopsy studies done on 37 patients (this number has now been extended to over 100 patients) dying in a state mental institution A very careful technique was developed for the measurement of the amount of intimal tissue present at 16 specified segments of the coronary arteries and 24 segments of the cerebral arteries involving the circle of Willis and its principal supply arteries The pathologic studies were done by Dr Malamud of the Langley Porter Neuropsychiatric Institute Statistical evaluation of the data showed that coronary and cerebral atherosclerosis are highly and significantly correlated (Pearson $R = + 0.59$ $P < 0.001$) This correlation indicates that the predictive value of serum lipoprotein measurements proven for coronary atherogenesis most likely also reflects the operation of a lipid metabolic factor in cerebral atherogenesis

Following these studies we investigated serum lipoprotein distributions in 88 male schizophrenic patients in two state mental institutions It was found that their lipoprotein patterns did not differ significantly from those of an age matched normal population from which we concluded that cerebral atherosclerosis is most likely not a significant etiologic factor in schizophrenia This is an interesting by product of human serum lipoprotein studies

WERTHESSEN I would like to ask specifically did you see these heavy lesions and the cerebral accidents only in those animals who were given ACTH and thiouracil or were they duplicated in animals given ACTH and that had their thyroids removed? In other words is this phenomenon specific for thiouracil?

ROSENFELD We have at no time used thyroidectomy and ACTH ACTH was used only prior to and/or with thiouracil and cholesterol The observations reported have been obtained from those animals who have received ACTH thiouracil and cholesterol I might add parenthetically that these animal were the animals who had consistently been the most hypercholesterolemic animals for the longest period of time

ADLERSBERG We know of course that the administration of ACTH and cortisone may be associated with thromboembolic phenomena in man I would like to know first whether central nervous symptoms were noticed in dogs that received cholesterol and propyl thiouracil without ACTH and secondly in those dogs re

Influence of Estrogens on Lipids and Atherosclerosis in Experimental Animals[1]

R Pick J Stamler and L N Katz

Cardiovascular Department Medical Research Institute Michael Reese Hospital Chicago Illinois

Our interest in the possible influence of estrogens on atherogenesis was first stimulated almost a decade ago by two problems which appeared to be of great importance for research in this field The first was What is the basis for the well known sex difference among middle aged persons in morbidity and mortality from coronary atherosclerosis? The second was What is more important for the development of clinically manifest atherosclerosis hypercholesterolemia or elevation of the cholesterol/phospholipid (C/P) ratio? Data forming the basis for the first question are illustrated in Figs 1–3 (2 8 13) The second problem stimulating our experimental studies with estrogens is illustrated in Figs 4 and 5 (1 12) Note the different effects of cholesterol feeding vs estrogen administration on the plasma C/P ratios in birds Cholesterol feeding results in a high C/P ratio In contrast estrogen administration leads to a predominant hyperphospholipemia with a low C/P ratio It therefore occurred to us that combining cholesterol feeding with estrogen administration would induce marked hypercholesterolemia with normal C/P ratio—thereby permitting experimental assessment of the role of hypercholesterolemia vs C/P ratio elevation in atherogenesis A series of experiments was therefore undertaken in cockerels combining an atherogenic diet high in cholesterol and oil plus estrogen administration The initial study was of the prophylactic type i e exhibition of the atherogenic diet and treatment with estrogens were started simultaneously in order to assess whether—in birds with marked hypercholesterolemia—both elevated C/P ratios and atherogenesis could be prophylactically inhibited In this particular experiment parenteral estradiol benzoate 1 mg per bird per day was given There was a marked feminizing effect reflected in the comb index (Fig 6) The anticipated effect on the C/P ratio was obtained (normally this ratio is 0 8 or lower in chicks) Thoracic aorta atherogenesis was not prevented by estrogen administration However estrogens induced a significant inhibition of coronary atherosclerosis Morphologically these plaques were generally pure atheroma without secondary changes cor

[1] Supported in part by grants from the Michael Reese Research Foundation the National Heart Institute (H2276) and the Chicago Heart Association

Established Investigator American Heart Association As of April 1 1958 Director Heart Disease Control Program Chicago Board of Health



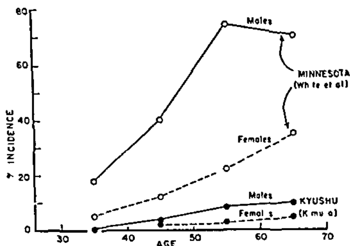


FIG 3 Incidence of high grade coronary sclerosis in consecutive autopsies United States versus Japan Note the gross sex difference for Americans (13) and its virtual absence for the Japanese (2) Note also the markedly higher incidence of severe coronary sclerosis in American males at all ages

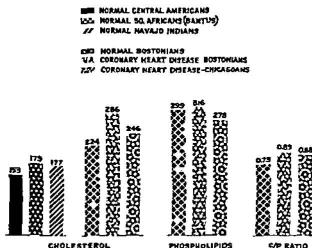


FIG 4 Serum lipids in middle aged normal males and males with coronary heart disease (12) Note the elevation of total serum cholesterol and C/P ratio of patients with myocardial infarction vs clinically healthy men of the same age Here and in subsequent figures where several parameters are presented in a single graph, each group of columns dealing with an individual variable is scaled separately

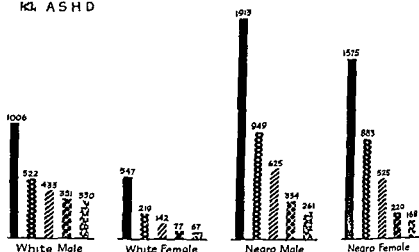


Fig 1 United States death rates 1950 age 45–54 Data from the U S National Office of Vital Statistics Death rates are per 100 000 population C-V R is cardiovascular renal A S + D H D is arteriosclerotic plus degenerative heart disease A S H D is arteriosclerotic heart disease Note the large sex difference in all categories particularly in the white population Here and in subsequent figures the values at the top of the column represent the actual observations in this case the death rates See reference (8)

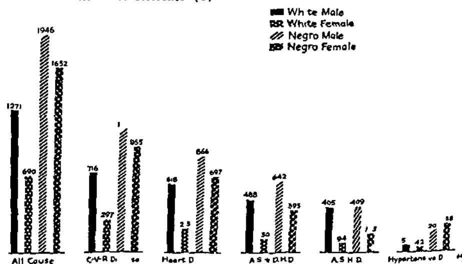
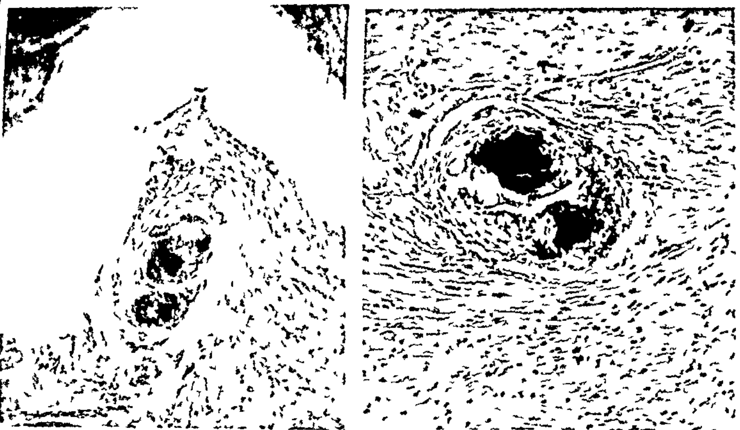


Fig 2 Death rates between the ages of 45 and 54 according to sex and race as tabulated in Chicago from 1951 to the present (8) See legend to Fig 1 Note again the large sex difference in all categories with the exception of the hypertensive diseases Note that this sex difference is especially large for A S H D in the whites



(A) (B)

Fig 7 Microscopic appearance of coronary lesions (A) Frozen section Sudan IV hematoxylin 100 × Note large intimal lipid containing plaque This bird was on a 1% cholesterol 5% cottonseed oil (1C O) diet for 5 weeks (B) Paraffin section hematoxylin eosin 100 × Note plaque with foam cells and large calcium deposit This bird was on a 1C O diet for 13 weeks

responding to degree one in Dr Russell Holman s classification of lesions (Fig 7) This is the usual finding in short term (5 week) experiments In experiments of longer duration fibrosis hyalinization and calcification are frequently found in these lesions In this experiment—it is worth re emphasizing—thoracic atherosclerosis was very little influenced

Data on the individual chicks in this experiment revealed additional findings of possible significance (Fig 8) (6) Thus all cholesterol fed

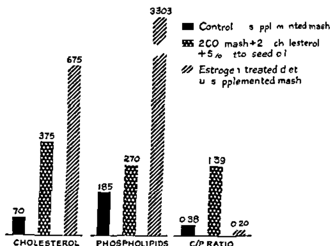


FIG 5 Effects of cholesterol oil feeding vs estrogen administration on plasma lipids of cockerels (12)

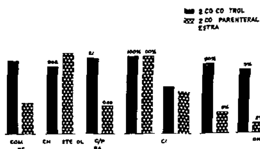


FIG 6 *Estrogen prophylaxis of coronary atherosclerosis in cholesterol fed* cockerels (6) The first pair of bars dealing with coronary lesions (90% and 28%) represents the incidence of lesions i e the per cent of birds with lesions The second set of bars (13% and 2%) is the coronary count an index of the extent of the atherosclerotic process It is derived by examining two representative Sudan IV stained sections of each bird s heart counting the total number of arteries and arterioles visualized and determining the per cent which exhibit normal morphology lipid infiltration and plaque formation The data in this and subsequent figures represent the per cent of these vessels with atherosclerotic plaques

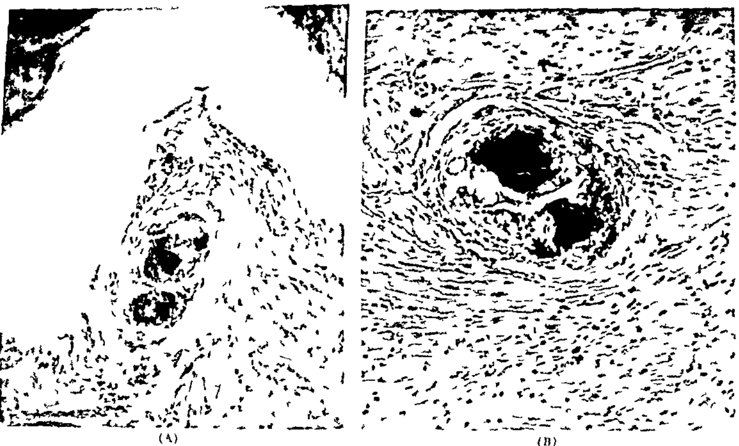


Fig. 7. Microscopic appearance of coronary lesions. (A) Frozen section. Sudan IV hematoxylin. 100 ×. Note large intimal lipid containing plaque. This bird was on a 1% cholesterol 5% cottonseed oil (1C O) *diet for 5 weeks*. (*B*) Paraffin section, hematoxylin eosin. 100 ×. Note plaque with foam cells and large *calcium deposit*. The bird was on a 1C O diet for 13 weeks.

animals had high C/P ratios and extensive coronary atherosclerotic lesions The cholesterol fed estrogen treated cockerels with normal C/P ratios were uniformly free of coronary lesions In contrast cholesterol fed estrogen treated chicks with elevated C/P ratios exhibited coronary lesions These data suggest—but do not prove—that in the chick at least the C/P ratio is related to the occurrence or prevention of coronary atherogenesis

This possibility is also suggested by data from experiments in rats and rabbits The former species on a potentially atherogenic diet re

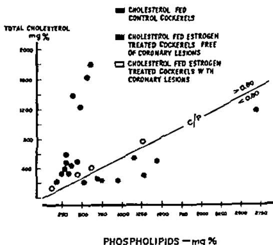


FIG 8 Effects of estrogens on plasma lipids and coronary atherogenesis in cholesterol fed cockerels (6) The diagonal line represents the normal C/P ratio

sponded to estrogens with enhancement of hyperphospholipemia lowering of C/P ratios toward normal values and inhibition of coronary atherosclerosis (3) In contrast cholesterol fed and cholesterol fed estrogen treated rabbits exhibited no difference in the serum lipid levels of the two groups (Fig 9) (12) Thoracic and coronary lesions were also equally prevalent in both groups Thus failure of estrogens to prevent C/P ratio elevation was associated with failure to inhibit coronary lesions The findings in these animal species would support the possibility that there is a relationship between the C/P ratio and the emergence or suppression of coronary atherogenesis

In further studies with cockerels it was soon demonstrated that a variety of *estrogens—oral and parenteral natural and synthetic*—induced the aforementioned effects on plasma lipids and coronary atherogenesis In all experiments thoracic atherogenesis—in contrast to coronary atherogenesis—developed without evidence of any inhibitory effect

of estrogens Thus the conclusion seemed inescapable that different biologic laws govern atherogenesis in different vascular beds

In subsequent experiments thoracic aortic lesions were sometimes more severe in the estrogen treated birds than in the cholesterol-oil fed controls In a recent experiment—in cockerels fed a low protein high fat high cholesterol diet and treated with estrogen—severely ulcerated abdominal aorta lesions were produced with hemorrhages in plaques dissecting aneurysms and (in one chick) possible thrombosis Full estrogen protection of the coronary vessels was maintained in this experiment

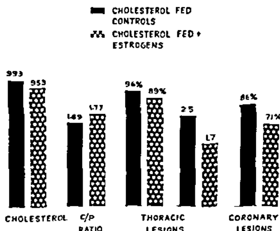


FIG 9 Effects of estrogen in cholesterol fed rabbits (series 1-4) (12)

A further experiment was undertaken in cockerels to assess the ability of estrogen to reverse previously induced coronary atherosclerotic lesions (Fig 10) This study was of the therapeutic type in its design The birds were fed the atherogenic diet from the age of 8 to 21 weeks The estrogen treated group received parenteral estradiol during the last 5 weeks of the experiment—with continued feeding of the high cholesterol high fat diet C/P ratios were reduced to normal in the estrogen treated group Thoracic aorta atherogenesis was severe in both groups Coronary atherogenesis was markedly diminished in the estrogen treated group (7) This experiment proved that estrogens are capable of therapeutically reversing coronary atherosclerosis previously induced by cholesterol feeding It confirmed previous observations from experiments of different design demonstrating that atherosclerosis is—within limits—a reversible lesion

A detailed histopathologic and histochemical study was carried out by Dr Rene Malinow (a former fellow of the Cardiovascular Depart

ment) and his colleagues residing in Buenos Aires Argentina These detailed studies revealed no evidence of residual lesions in the coronary vessels Therefore estrogens are undoubtedly capable of reversing both the lipid and the fibroblastic components of early atherosclerotic plaques In a small number of vessels however a slight calcium deposition was found in the intima—a residuum of previous atherosclerosis which did not effect encroachment upon the lumen of the vessel

All these experiments were carried out in cockerels receiving a large daily dose of parenteral or oral estrogen This dosage had been arrived

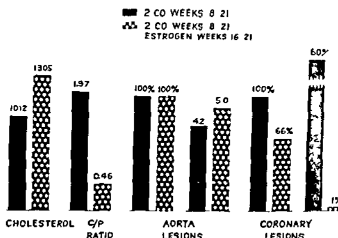


FIG 10 Estrogen induced reversal of coronary atherosclerosis in cholesterol fed cockerels (7) One group of 10 animals not shown in the figure was sacrificed at 16 weeks of age prior to the institution of estrogen administration in the experimental group One hundred per cent of these birds had severe extensive coronary lesions

at based on dose response studies evaluating the amount of estrogen required to produce in cockerels the plasma lipid patterns of mature estrogen secreting egg laying hens It seemed to be in order to elucidate *whether this effect of exogenous estrogens constituted a pharmacological action in cockerels* Experiments were therefore undertaken to evaluate whether the physiologic endogenous estrogen secretion of the egg laying hen protected the female chick from cholesterol induced atherosclerosis A preliminary study demonstrated that immature male and female chicks were equally susceptible to coronary atherogenesis consequent upon cholesterol feeding (12) In contrast mature estrogen secreting egg laying hens exhibited a marked resistance to coronary atherogenesis when fed mash supplemented with cholesterol and oil (Fig 11) However these hens exhibited lower plasma cholesterol

levels compared with age matched cockerels fed the same diet Consideration was therefore given to the possibility that the laying hen might be protected against coronary atherogenesis by disposing of some dietary cholesterol via egg laying A group of oviduct ligated hens was therefore included in this experiment (Fig 11) (11) These oviduct ligated hens were also resistant to cholesterol induced coronary atherogenesis C/P ratios in both female groups were very low a typical estrogenic effect—in this case a resultant of the physiologic endogenous estrogen secretion by the ovary This endogenous protection against coronary atherogenesis was lost by complete ovariectomy (Fig 12) (5) It was concluded that the protection of the coronary arteries against cholesterol induced atherosclerosis was due to the normal endogenous estrogen secretion of the hen

Estrogen administration was later combined with a number of other hormones in prophylactic type experiments in cholesterol fed chickens When estrogen and androgen were simultaneously given to birds on a potentially atherogenic diet feminization of secondary sex characteristics was prevented (Fig 13) (9) However estrogenic C/P ratios and protection of coronary arteries were maintained This is in contrast to the results on serum lipids lipoproteins in the human wherein combined androgen estrogen results in masculine patterns of serum lipids lipoproteins

In a further experiment estrogen administration and cholesterol feeding were combined with hydrocortisone administration producing a corticoid diabetes (Fig 14) (10) Again complete estrogen effect on coronary atherogenesis was obtained

In a subsequent study estrogen administration was combined with insulin administration in cockerels on high cholesterol high fat diet (Fig 15) This study was undertaken because of the well known clinical fact that diabetic females lose the usual immunity of their sex to coronary atherosclerosis The hypothesis leading to this experiment was that insulin treatment may by some mechanism increase susceptibility to atherogenesis in diabetic women Cholesterol fed cockerels given insulin plus estrogen exhibited a tendency to higher C P ratios than the estrogen treated controls (Fig 15) (12) There was also partial loss of protection against coronary atherogenesis in these birds

In a further experiment it was shown that a euthyroid state is necessary for estrogens to exert their protective action on the coronary arteries (Fig 16) (4) Hypothyroidism induced by thiouracil feeding or by thyroidectomy and I^{131} administration partially counteracted the ability of estrogens to exert their beneficial effects on the coronary arteries in cholesterol fed cockerels

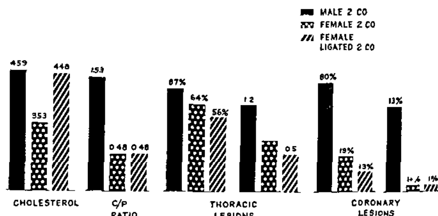


Fig 11 Effects of oviduct ligation in mature egg producing estrogen secretin hens fed a cholesterol oil mash (11)

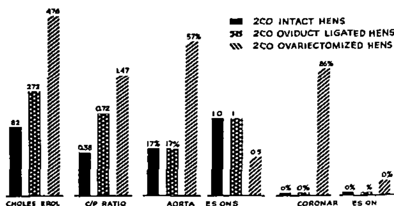


Fig 12 Effects of ovariectomy on plasma lipids and atherogenesis in cholesterol fed hens S37 and 43 age 33–44 weeks (5)

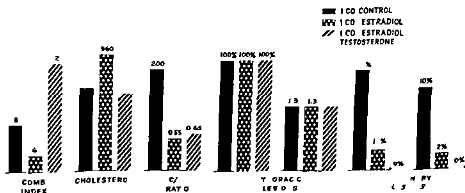


Fig 13 Effects of estrogen plus androgen in cholesterol fed cockerels (9)

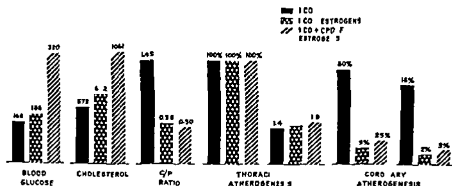


Fig 14 Failure of corticoid diabetes to abolish estrogen prophylaxis of cholesterol induced coronary atherogenesis in cockerels (10)

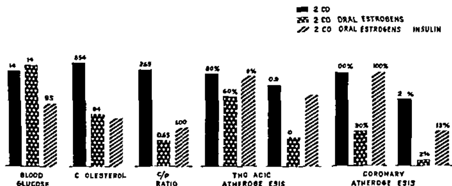


Fig 15 Insulin counteraction of estrogen anti atherogenesis in cholesterol fed cockerels (12)

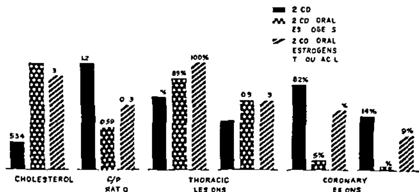


Fig 16 Thiouracil counteraction of estrogen anti atherogenesis in cholesterol fed cockerels (4)

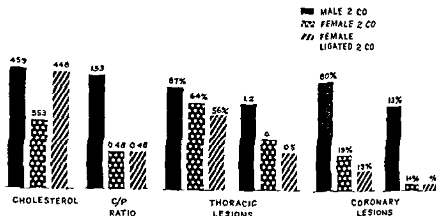


Fig 11 Effects of oviduct ligation in mature egg producing estrogen secreting hens fed a cholesterol oil mash (11)

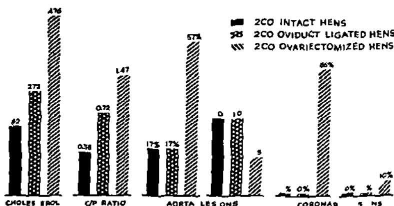


Fig 12 Effects of ovariectomy on plasma lipids and atherogenesis in cholesterol fed hens S37 and 43 age 33–44 weeks (5)

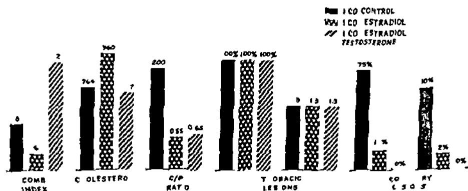


Fig 13 Effects of estrogen plus androgen in cholesterol fed cockerels (9)

C/P ratio that you observed It could be that the blood vessel is busy making phospholipids and pouring them out into the circulation where you can measure them This would fit you see with your idea that by getting the phospholipids into the blood the cholesterol per se is rendered inactive

As to Dr Adlersberg s work the aorta *in vitro* responds to hydrocortisone by a terrific increase in turnover of the lipids It may be that what is going on in the response to hydrocortisone is not a changing of its permeability but a conversion into a status where the vessel handles the lipids which are imposed upon by the circulation Now mind you I am merely speculating here but I would like to point out that one can take this position and reasonably interpret the data from the point of view that the blood vessel per se is responding to the hormones that are being administered

PICK Thank you Dr Werthessen It is quite possible I must say that after many years of working with estrogens we have not come closer to the mechanism of their action but I was careful to point out that it need not be the C/P ratio but the C/P ratio may be an expression of something that is going on elsewhere There is also some slight evidence perhaps that maybe it has to do with increased mast cell production stimulated by estrogen administration In the histopathological studies that I mention d an increased number of mast cells was seen in the walls of the coronaries of the treated animals In one experiment we tried by a very poor means I must say to block the reticuloendothelial system by giving thorotrast Nobody knows how complete such blockage is and we obtained negative results in that full estrogen protection was maintained in the chicks It might very well be that it works through the blood vessel wall but why only in the coronaries and not in the aorta? This is always the difficulty for every explanation that is proposed

WERTHESSEN I would like to raise a point on the difference between the coronaries and the aorta Is there a possibility here that by changing the amount of cholesterol in the diet and the stress if you want to call it that imposed by cholesterol on the vessel that you might bring the aorta into a condition where it could respond to estrogen? After all you have used essentially only one dose of estrogen and cholesterol One of the tenets of endocrinology is that changes in effect are observed over a dose range It may be that by working up and down the scale here you can get the coronary and the aorta to show similar effects I think this should be tried We did see in some of the walls that we didn t report last night that it can take a blood vessel several days to respond to estrogen There is distinct indication here that the enzyme system of the aorta changes during estrogen administration This could fit with your observation of the development of mast cells

PICK Dr Werthessen as Dr Stamler pointed out this mornin giving estrogens alone can produce some aortic lesions in cockerels So I don t think we have a good model to try this out in the bird

HOLMAN In keeping with the questions that Dr Werthessen just raised there is another possibility The coronary artery is predominantly a muscular artery in contrast to the aorta which is predominantly an elastic artery I am wondering if you have checked other muscular arteries such as the subclavians and femorals

PICK The abdominal aorta only It is also a muscular artery and in some experiments particularly if we extend the time of the experiment the abdominal aorta gets very severely involved in estrogen treated animals

This series of experiments yielded extensive support for the concept that estrogenic secretion is a key factor responsible for resistance to coronary atherogenesis among women in populations habitually ingesting a potentially atherogenic diet Together with extensive clinical pathologic and epidemiologic observations it formed the animal-experimental basis for undertaking a long term clinical evaluation of the possible efficacy of estrogens in patients with clinical coronary heart disease In behalf of our group Dr Jeremiah Stamler discusses our experiences in this clinical investigation in a subsequent paper

ACKNOWLEDGMENTS

We are greatly indebted to Dr Edward Henderson of the Schering Corporation Bloomfield New Jersey for generous supplies of estradiol benzoate (Progynon B) and testosterone to Dr John B Jewell of Ayerst McKenna and Harrison Limited New York City for generous supplies of Premarin and to Dr Kenneth Kohlstaedt of the Lilly Laboratory for Clinical Research Indianapolis Indiana for generous supplies of insulin and thiouracil

REFERENCES

1 Katz L N and Stamler J Experimental Atherosclerosis C C Thomas Springfield Illinois 1953
2 Kimura N *in* World Trends in Cardiology I Cardiovascular Epidemiology (A Keys and P D White eds) p 22 Hoeber Harper New York 1957
3 Moskowitz M N Moskowitz A A Bradford W L Jr and Wissler R W A M A *Arch Pathol* 61 245 (1956)
4 Pick R Stamler J and Katz L N *Circulation Research* 5 510 (1957)
5 Pick R Stamler J and Katz L N *Circulation Research* 5 515 (1957)
6 Pick R Stamler J Rodbard S and Katz L N *Circulation* 6 276 (1952)
7 Pick R Stamler J Rodbard S and Katz L N *Circulation* 6 858 (1952)
8 Stamler J *J Natl Med Assoc* 50 161 (1958)
9 Stamler J Pick R and Katz L N *Circulation Research* 1 94 (1953)
10 Stamler J Pick R and Katz L N *Circulation* 10 247 (1954)
11 Stamler J Pick R and Katz L N *Circulation* 10 251 (1954)
12 Stamler J Pick R and Katz L N *Ann N Y Acad Sci* 64 596 (1956)
13 White N K Edwards J E and Dry T J *Circulation* 1 645 (1950)

DISCUSSION

WERTHESSEN The question I am going to ask I trust will stimulate a bit of discussion I would like to make the suggestion that the results observed in these experiments and in earlier ones reported by Dr Adlersberg are responses of the blood vessels to the hormones It so happens that in both cases the effects observed are interpretable in the light of what we have seen in excised *in vitro* aortas The effect of estrogen *in vitro* is to increase incorporation into the phospholipids of the aorta In view of the total size of the vascular organs as Dr Holman demonstrated it may very well be especially if Dr Zilversmit is right and the phospholipids do not move from blood into the aorta that the phospholipids that you observe come from the blood vessels move into the blood and produce the favorable

would seem that these values are increased primarily through endogenous means and that these and other serum and artery changes are due to factors other than of a dietary nature A method which will measure this response quantitatively is useful in the study of atherosclerosis

TABLE B

EFFECT OF DEPO ESTRADIOL CYCLOPENTYLPROPIONATE (ECP) ON YOUNG COCKERELS

ECP (I M) (mg)	Serum Albumin (cm)	Serum Globulin (cm)²
0 0	5 0	7 4
2 5	4 2	11 8

Results included in Table B summarize serum albumin and globulin data obtained with a relatively large number of normal and estrogen treated birds at the 2 50 mg level The mean value for the serum globulin of the treated birds is significantly higher statistically ($P < 0\ 001$) than that for the normal controls For serum albumin the mean values for the positive and negative controls are significantly different statistically ($P = 0\ 01$)

TABLE C

COMPARISON OF EFFECT OF DEPO-ESTRADIOL CYCLOPENTYLPROPIONATE (ECP) AND CHOLESTEROL ON YOUNG COCKERELS EXPERIMENTAL PERIOD—56 DAYS

ECP (I M) mg / bird/ 10 days	Cholesterol added to diet (%)	Degree of athero sclerosis (% area)	Serum Cholesterol Total (mg %)	Serum Cholesterol Free/ total (%)	Serum Phospho lipid phos phorus (mg %)	Serum Total polysac charides (mg %)	Serum Lipoproteins mg % cholesterol in α	Serum Lipoproteins mg % cholesterol in β
0 0	0 0	0 02	100	22	7	72	70	38
0 0	0 5	16 2	227	26	6	76	63	80
5 0	0 0	22 8	383	58	46	117	2	710
5 0	0 5	19 5	839	37	48	120	—	—

Data included in Table C make possible a comparison of results obtained with estrogen cholesterol and estrogen plus cholesterol treatment of cockerels The experimental period for these studies was 56 days The estrogen treatment was intramuscular The dosage was equivalent to 5 0 mg administered at the beginning of each 10 day period The atherogenic diet contained 0 5% added cholesterol

Attention is called to the relatively small difference in the degree of athero sclerosis observed for each of the three experimental groups There is considerable difference in the serum cholesterol the combined treatment resulting in more than additive amounts for the other two groups The free to total cholesterol is con siderably higher for the estrogen treated group The intermediate value for the combined treatment indicates relatively more esterified cholesterol than the estrogen treated group and relatively less than for those treated with cholesterol only Phospholipid phosphorus and total polysaccharides are elevated in both cases of

CALDWELL We have studied quantitatively the atherosclerotic effect of estrogenic material on young birds Four week old white Leghorn cockerels have b en treated with Depo estradiol cyclopentylpropionate We have determined the degree of atherosclerosis as manifested grossly in the aorta and other large arteries in addition to chemical changes reflected in the blood serum The effect has been observed over varying periods of time and with graded dosage levels of the estrogenic material

TABLE A

EFFECT OF DEPO ESTRADIOL CYCLOPENTYLPROPIONATE (ECP) ON YOUNG COCKERELS

ECP (I M) (mg)	Degree of atherosclerosis (Plus values)	Serum Cholesterol Total (mg %)	Serum Cholesterol Free/total (%)	Serum Phospholipid phosphorus (mg %)	Serum Total polysaccharides (mg %)	Serum Lipoproteins mg % cholesterol in α	Serum Lipoproteins mg % cholesterol in β
0 00	0 0	112	22	6	68	91	40
0 156	0 2	105	25	7	78	90	38
0 625	0 2	103	21	7	63	81	46
1 25	2 5	110	23	8	73	50	92
2 50	2 8	216	30	15	92	17	233
5 00	2 4	451	57	54	1°0	7	565
7 50	2 0	378	66	60	1 55	2	664

Data included in Table A summarize results using the indicated amounts of estrogens The experimental period for these studies was 7 days At the beginning of this period each bird was administered the Depo-estrogen I M They were fed a chick growing mash ad libitum At the close of the 7 day period they were rendered unconscious by electrocution the blood was removed directly from the heart and the aorta and other large arteries were dissected for gross examination We did not study the coronary arteries

Your attention is called first to the results for the negative control groups and for the do age levels of from 0 156 to 1 25 mg given at the beginning of the 7-day period The values for the total cholesterol and the free to total ratio for phospholipid phosphorus and total polysaccharides are essentially the same at these dosage levels as for the control groups However even at the lowest dosage level a trace of atherosclerosis was observed This became quite pronounced at the 1 25-mg level Also the α lipoprotein fraction has decreased and the β lipoprotein fraction has increased significantly at this level These data suggest that changes in the arteries and serum lipoproteins are among those most readily affected by this treatment

At the 2 50 mg dosage level definite changes have occurred in all of the measurements indicated And as the amount of estrogenic material is increased to 5 0 and 7 5 mg these changes with the exception of the degree of atherosclerosis generally speaking become greater

The 2.50-mg level is the smallest amount we have found to result in definite changes in all of these measurements Therefore it has been used in the preparation of an experimental method based upon estrogen induced atherosclerosis Since the birds are fed a chick growing mash diet without additional cholesterol the enhanced serum cholesterol values certainly are not due to exogenous sources It

Many people have been talking about C/P ratios and have also commented on rises in phospholipids in connection with rises in cholesterol Many years ago I described a correlation between plasma lipids which was deduced from observations made on cholesterol fed animals This relationship could be expressed by a mathematical equation Subsequently I collected all published data I could lay my hands on relating to all species and surprisingly I found that these data fitted the experimental curves deduced from cholesterol fed animals If we plot as the abscissae the values of plasma free cholesterol (x) not total cholesterol and on the ordinate we plot the phospholipids or nonphospholipid fatty acids (y) then we get a curve which can be expressed by the general equation $y = ax^b$ It is many years since I described this and I do not remember the numerical values of the constants The constants a and b have characteristic values for either the interrelationship of free cholesterol to phospholipid or free cholesterol to nonphospholipid fatty acids This interrelationship can be applied to all human conditions pathologic or normal to all species ranging from cockerel rat, rabbit sheep dog to any of the animals that data have been published for I think it probable that if in the course of the various treatments that have been described (resulting in plasma lipid changes) the free cholesterol values were plotted against the concentration of phospholipids this same mathematical relationship would be found

PICK I am sorry Dr Popjak that I did not bring any slides of lesions but I assure you the lesions are intimal in nature they are plaques mostly of the early lesions in the 5 week experiment that are like Dr Holmans lesion 1 they are pure atheroma If the animals are kept longer on diets there will be a significant amount of fibrosis and the lipids will be in the deeper media near part of the lesion There is also some lipid infiltration particularly in the larger lesions in the media but the lesion itself is intimal in nature We had in several experiments determined ester and free cholesterol but I do not remember the data Maybe Dr Stamler does

STAMLER As Dr Caldwell showed estrogens have the unique property of increasing plasma free cholesterol and the ratio of free to total (F/T) cholesterol In fact they are to date the only agents known to alter this ratio in chicks They also *markedly reduce the α lipoprotein concentration in this avian species—an* effect opposite to that exerted by estrogens in man It is therefore almost a certainty that Dr Popjaks equation would hold very well i e there is a close proportional ty between increase in plasma cholesterol and phospholipids under estrogen influence

MARMORSTON I am very grateful to Dr Popjak for this picture We found that irrespective of the three clinical groups myocardial infarction well and sick controls and irrespective of age the cholesterol phospholipid regression equation was the same

CALDWELL In connection with Dr Stamlers remarks I want to state that my principal purpose in presenting this material is the quantitation of observations some of which have been known for some time in a general way

estrogen treatment Also with estrogen treatment there is a marked decrease in α and increase in β lipoprotein values These data emphasize major differences observed in the estrogen cholesterol and combined treatment of young cockerels with the study continued during a 56 day period

FURMAN In view of Dr Pick s observations I would like to take this opportunity to report some unpublished observations that my associate Dr Loyal L Conrad and I have been able to make in a woman who recently died after an extensive period of hypercholesterolemia and hyperphospholipemia secondary to pericholangiolytic biliary cirrhosis with extensive xanthomatosis This woman was 30 years of age at death and during the previous 8 years her serum cholesterol concentration had ranged between 450 and 2800 mg % Serum cholesterol and phospholipid circulated solely as $-S_{1\ 1}$ 25–40 and 40–70 (S_f 0–12 and 12–20) lipoproteins There were no $-S_{1\ \cdot 1}$ 0–20 (alpha) lipoproteins present in her serum Of interest is the fact that the degree of hyperphospholipemia was equivalent to or relatively greater than the hypercholesterolemia so that the C/P ratio was constantly 1 or less At autopsy the coronary arteries were clear The aorta on the other hand was extensively damaged with atherosclerotic plaques Calcification was present in the abdominal aorta which we had detected by X ray 3 years previously This dichotomy in atherosclerotic involvement of coronary arteries and aorta lends some weight to the suggestion that coronary and aortal atherosclerosis deserve consideration as two disease entities The role of the phospholipids and the significance of the C/P ratio in atherogenesis while still unclear nevertheless must continue to receive serious study

For those of us who enjoy speculation about the role of filtration as a factor in atherogenesis it is of interest to point out—and as far as I have been able to determine this is a previously undescribed finding—that on examination of the brain extensive deposition of crystalline material presumably cholesterol was noted in the choroid plexuses with marked distortion of the histologic picture in this region

STAMLER Dr Caldwell is it not lipid infiltration of the aorta which you are observing without atherosclerosis? Is it correct to call this atherosclerosis? If aorta atherosclerosis is induced by estrogens in such short term experiments this would be a finding quite different from that of Lindsay and Chaikoff and of our group In our experience estrogen treated chicks on a plain mash diet—without a cholesterol supplement—develop thoracic aorta lesions only after months of hormone-induced endogenous hypercholesterolemic hyperlipemia Of course for reasons Dr Pick clearly delineated this regimen induces aorta lesions only never coronary lesions

CALDWELL I prefer to postpone a more definitive answer to your question until that phase of the problem has been studied further

MILCH I should like to point out that after I^{131} administration to the dog neither aortal nor coronary atherosclerotic lesions can be demonstrated in spite of the fact that such an animal maintains blood levels of cholesterol and β lipoproteins far in excess of anything ordinarily seen in the human On the other hand although lipid phosphorus levels are also greatly increased the cholesterol phospholipid ratio is if anything also increased

POPJAK May I ask if anyone has any sections or photographs to show of the coronary lesions in these birds so that we really know what we are talking about?

I would like to comment about the interrelationship between plasma lipids

Many people have been talking about C/P ratios and have also commented on rises in phospholipids in connection with rises in cholesterol Many years ago I described a correlation between plasma lipids which was deduced from observations made on cholesterol fed animals This relationship could be expressed by a mathematical equation Subsequently I collected all published data I could lay my hands on relating to all species and surprisingly I found that these data fitted the experimental curves deduced from cholesterol fed animals If we plot as the abscissae the values of plasma free cholesterol (x) not total cholesterol and on the ordinate we plot the phospholipids or nonphospholipid fatty acids (y) then we get a curve which can be expressed by the general equation $y = ax^b$ It is many years since I described this and I do not remember the numerical values of the constants The constants a and b have characteristic values for either the interrelationship of free cholesterol to phospholipid or free cholesterol to nonphospholipid fatty acids This interrelationship can be applied to all human conditions pathologic or normal to all species ranging from cockerel rat rabbit, sheep dog to any of the animals that data have been published for I think it probable that if in the course of the various treatments that have been described (resulting in plasma lipid changes) the free cholesterol values were plotted against the concentration of phospholipids this same mathematical relationship would be found

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Effect of New Steroids on Blood Lipids

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The blood lipid pattern in animals and man can be altered by the administration of estrogens and abnormal lipid values in subjects with atherosclerosis or in cholesterol fed animals can be returned toward or to normal by such treatment Katz and associates have reported that the administration of estrogens to cholesterol fed cockerels has a beneficial prophylactic effect as well as a therapeutic effect on coronary atherosclerosis (13) It was also shown by Pick *et al* (18) that depression of the cholesterol phospholipid ratio was associated with inhibition of the developing coronary lesions Similar changes may be produced by estradiol benzoate in rats (14) Other evidence has accumulated demonstrating that myocardial infarction occurs much more frequently in men than in women particularly under the age of forty (3 13) It is not unreasonable then to suppose that the effects of estrogens on lipid metabolism may be of therapeutic value in coronary artery disease Androgens on the other hand may have an adverse effect

The administration of estrogens to patients with coronary heart disease and abnormal lipid patterns causes significant changes in serum lipids returning the values to or towards normal (1 2 3 16 17 19 20 22 24) Although estrogens can change serum lipids it is not known whether such treatment would ultimately inhibit the atherosclerotic process Further the incidence of estrogenic side effects is high particularly in middle aged men (20 24) In attempts to avoid the undesirable estrogenic action investigators have employed very low doses of estrogens intermittent estrogen therapy or estrogen androgen combinations (1 16 22) Androgens however can antagonize the effect of estrogens on blood lipids (17 22) and combinations studied to date have not been useful

The most promising hormonal approach in this phase of atherosclerosis is the preparation of steroid derivatives that retain lipid shifting effects but that have a lower estrogenic potency (9) Several such steroids showing good lipid activity combined with relatively low estrogenic potency have been studied in detail by Cook and associates (5 7 15) The prediction of estrogenic side effects in man from such data is discussed in a separate paper In the present report certain structure activity relationships of steroid derivatives will be discussed and data on three compounds which differ qualitatively and quantitatively in their actions will be presented

METHODS

Lipid Studies One day old Hy line cockerels were reared in a battery brooder and fed chick starter mash until 6½ weeks of age During test periods they were fed an atherogenic mash diet containing 2% cholesterol (Cholesterin Wilson Co) and 5% cottonseed oil Total plasma cholesterol was determined by the method of Zlatkis *et al* (25) Lipid phosphorus was determined by Sperrys method (23) and multiplied by a factor of 25 to give the amount of total phospholipid Compounds were dissolved in corn oil and administered subcutaneously except when oral effectiveness was determined

In acute studies animals were placed on the atherogenic diet and compound administered subcutaneously or orally once a day for 3 days Control birds received equivalent doses of corn oil Twenty four hours after the last dose blood samples were taken from the wing vein in heparinized syringes for determination of plasma cholesterol and phospholipid

In the chronic experiments cockerels were fed the atherogenic diet for 8–10 weeks the compound being administered subcutaneously once a day Plasma cholesterol phospholipids body weight and food consumption were determined at intervals during the study All birds were sacrificed with intravenous sodium pentobarbital The aortas and hearts were removed for gross and histological examinations by methods previously reported (6) Other organs including the liver and testes were removed and fixed in formalin for histological examination The size of the combs length plus height (comb index) was determined for all birds at intervals during the experiments

Estrogenic Activity Estrogenic potency was determined in mice using a modification of the uterine weight method of Rubin *et al* (10 21) Groups of 8 to 10 immature mice 22–25 days of age were used at each dose level Estrogenic activity was also determined in rats using the vaginal smear technique employing the procedure of Edgren and Calhoun (11) The smears were evaluated according to the method of Biggers and Claringbold (4) wherein the absence of leukocytes is considered a positive response Estrogenic potencies given in the tables are based on doses necessary to produce a 100% change in uterine weight of treated animals when compared with simultaneous controls

RESULTS

A *Effects of Standard Compounds*

In all of the studies to be reported estrone was used as a standard and assigned a potency of 100% in the lipid test on cockerels and in the

estrogenic assays Thus for this compound the ratio of lipid effect to estrogenic potency is 1 Other common estrogens can affect lipid metabolism in the cockerel but the potency of these compounds varies (Table I) When compared with estrone estradiol and its derivatives and diethylstilbestrol have a lipid/estrogenic ratio of less than 1 indicating that relatively their estrogenic effect is greater than their lipid effect Estriol is the only standard estrogen we have studied that shows ratios greater than 1 these ratios were in the range of 6–10 as compared with estrone

TABLE I
LIPID VERSUS ESTROGENIC ACTIVITY

	Lipid effect (A)	Estrogenic potency: Mouse uterine (B)	Estrogenic potency: Rat vaginal (C)	Ratio A/B	Ratio A/C
Estrone	100	100	100	1 0	1 0
Estradiol	131	370	650	0 4	0 2
Estradiol benzoate	326	476	418	0 7	0 8
Ethinylestradiol	87	833	1020	0 1	0 1
Estriol	73	11 9	7 4	6 1	9 9
Diethylstilbestrol	163	625	478	0 3	0 4

It should also be pointed out that the effects of estrogen in cockerels differ in part from the effects obtained in man In both cockerels and man estrogens will decrease the C/P ratio In cockerels however the administration of estrogen may increase the blood cholesterol level The relative increase in phospholipids is greater than any change in cholesterol resulting in a decrease in the C/P ratio In man androgens will increase serum cholesterol whereas in cockerels androgens do not influence serum cholesterol or phospholipids

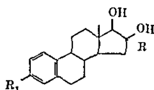
B Newer Steroids

3 16 Substituted Estriol Derivatives In this series of compounds synthesized by Dr D A Tyner substitutions were made at the 3 and 16 position of estriol (Table II) The most potent lipid effect was obtained with the compound having a 16 methyl group and a 3 methyl ether This compound has a lipid effect that is 31% that of estrone Estrogenicity is 1 6% that of estrone giving a lipid/estrogenic ratio of 19 This substance will later be referred to as SC 6924 or Manvene Other modifications in the structure did not increase lipid potency or significantly increase the lipid/estrogenic ratio

5(10) Unsaturated Compounds A series of 17 alkyl derivatives with the double bond in the 5(10) position synthesized by Dr F B

TABLE II

LIPID VERSUS ESTROGENIC ACTIVITY OF ESTRIOL DERIVATIVES

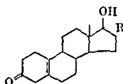


R_1	R	Other	Lipid	Estrogen	Ratio
OMe	H		13	1	13
OMe	H	1 Me	< 5	—	—
OMe	Me		31	1 6	19
OH	Me		23	c 1 0	23
OMe	Me	1 Me	< 5	c 1 0	—
OMe	Et		— 5	—	—

Colton was also studied The compound with the highest lipid/estrogenic ratio is the 17 methyl derivative which has a lipid effect of 24% that of estrone and an estrogenic effect approximately 1% that of estrone (Table III) In addition the compound also has an androgenic effect equivalent to 5% that of testosterone propionate This compound designated SC 6582 will be mentioned again in comparison with SC 6924 Substitutions other than the 17 methyl group did not increase lipid effects or produce a more favorable lipid/estrogenic ratio

TABLE III

LIPID VERSUS ESTROGENIC ACTIVITY OF 5(10) ESTRENONES



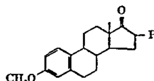
R	Lipid	Estrogen	Ratio	Androgen
H(3-OH)	27	10	3	5
Methyl	24	1	24	5
Ethyl	< 10	< 0 04	—	1
Ethinyl	26	7	4	< 1

16 Halogenated Estrone Derivatives Halogenation in the 16 position can also influence lipid potency (15) In the series of 3 methoxy derivatives the best lipid/estrogenic ratio was obtained by substitution of a 16α-chlorine Lipid effect is nearly equivalent to that of estrone and estrogenicity is quite low (Table IV) The chlorine derivative

SC-8246 will be further compared with the most active compounds in the other chemical series mentioned above

TABLE IV

LIPID VERSUS ESTROGENIC ACTIVITY OF 16-HALOGENATED ESTRONE DERIVATIVES



R	Lipid	Estrogen	Ratio
Cl	90	0 79	114
I	143	1.8	95
Br	2[illegible]	1.3	20

C Comparison of SC-6924 SC-6582 SC-8246

Each of the chemical series discussed above shows lipid activity but the biological effects of active compounds differ qualitatively or quantitatively from one another It is of interest then to compare in more detail the lipid, estrogenic, and androgenic properties of these substances the structures of which are compared with estrone in Fig 1

Estrogenic Activity All three of the compounds may be called weak estrogens for their estrogenic effects in the standard assay procedures are much less than the effect of estrone and related substances The dose response curves for estrone and SC-6924 in the mouse uterine

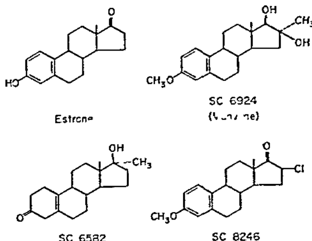


FIG 1 Structure of estrone and lipid-shifting steroids

weight tests are shown in Fig 2 Estrone gives a typical steep dose response curve whereas the dose response curve for SC 6924 is significantly shallower and is not parallel to that for estrone At our comparative response level SC 6924 is approximately 1 6% as estrogenic as estrone whereas at the highest dose level employed potency is 0 1% that of estrone (5)

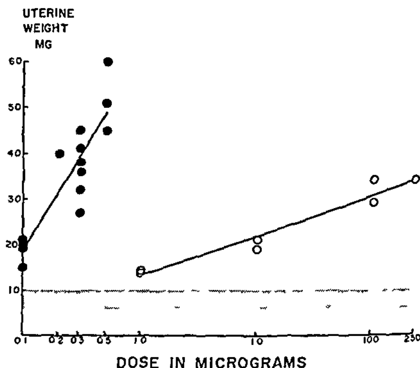


Fig 2 Effect of SC 6924 and estrone on uterine weight in immature female mice Closed circles = estrone open circles = SC-6924 Each point represents the mean of 8 to 10 animals Regression lines fitted by method of least squares Shaded area indicates range of uterine weight of untreated animals

Using the nomenclature of Huggins and Jensen (12) SC 6924 may be called an "impeded estrogen The term "impeded estrogen means that such compounds produce a gradual increase in uterine growth over a broad range of dosages in contrast to the steep growth increment produced by normal estrogens over a narrow dose range They reported that estriol and certain other compounds were impeded estrogens and noted that compounds with oxygenated functions at position 6 or 16 of the nucleus would often antagonize the uterine growth response to estrone Edgren (10) has studied a series of natural and synthetic steroids finding that there is a series of at least three categories of

slopes of dose response curves for uterine growth are not necessarily correlated with 6 or 16 oxyge

The compound SC 6582 gives a uterine weigh characteristic of an impeded estrogen (Fig 3) similar to that of SC 6924 Inasmuch as these do not parallel to that of estrone it is difficult to the estrogenic potency of such steroids At the level SC 6582 has about 0 9% the estrogenic poten at the highest dose level employed estrogenicity estrone (7)

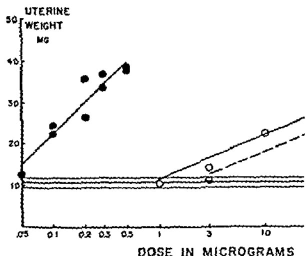


Fig 3 Effect of SC-6582 and estrone on uterine w mice Closed circles = estrone open circles = SC 65 neously half filled circles and broken line = SC-6582 ad Each point represents the mean of 8 to 10 animals Sh standard error limits of concurrent control animals

The last compound to be discussed SC 8246 s dose response curve characteristic of normal estr

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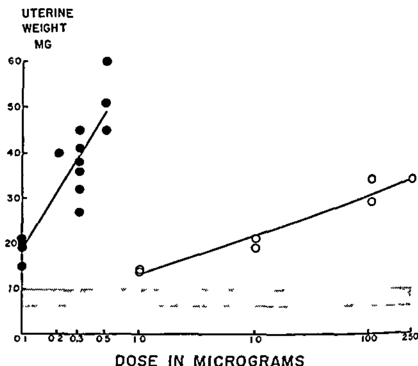


Fig 2 Effect of SC-6924 and estrone on uterine weight in immature female mice Closed circles = estrone open circles = SC 6924 Each point represents the mean of 8 to 10 animals Regression lines fitted by method of least squares *Shaded area indicates range of uterine weight of untreated animals*

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TABLE V

EFFECTS OF SC 6582 AND TESTOSTERONE PROPIONATE ON BODY WEIGHT SEMINAL VESICLES VENTRAL PROSTATE AND LEVATOR ANI WEIGHT

Compound	Total dose (mg)	Body weight Initial (g)	Body weight Final (g)	Body weight Gain (g)	Seminal vesicles (mg)	Ventral prostate (mg)	Levator ani (mg)
Controls	—	150	187	37	7 5	9 8	60 5
SC 6582	1 0	149	172	23[a]	11 9[a]	16 4[a]	67 3
	2 0	152	169	17	17 4[a]	15 1[a]	88 0
	5 0	151	161	10[a]	29 3[a]	21 0[a]	106 4[a]
Testosterone propionate	0 05	148	182	34	11 2[a]	15 0[a]	61 2
	0 2	153	185	32	43 0[a]	47 2	87 3

$P < 0.01$ that treated mean = control mean groups of 8 rats were used at each dose level

weight determined From the data shown in Table V the androgenic and myotrophic potencies of SC 6582 were calculated to be approximately 5% that of testosterone propionate It was hoped that this small degree of androgenicity might serve to offset some of the undesirable estrogenic side effects of such steroids without however, blocking the desirable effects on blood lipids It is also of interest that SC 6582 inhibited weight gain Such an effect is a catabolic activity common to estrogens and SC 6582 produced a myotrophic response despite the simultaneous catabolic effect

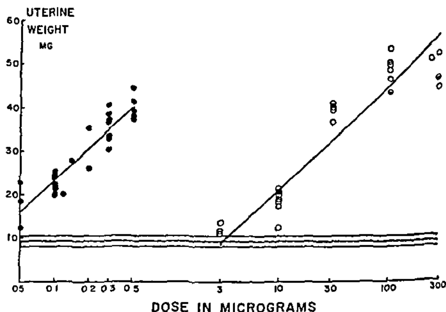


FIG 4 Effect of SC 8246 and estrone on uterine weight in immature female mice Closed circles = estrone open circles = SC 8246 administered subcutaneously half filled circles = SC-8246 administered intragastrically Each point represents the mean of 8 to 10 animals Shaded area represents two standard error limits of concurrent control animals

Acute Effects on Blood Lipids SC 6924 was administered in doses of 5 10 20 and 40 mg per kg to cockerels fed the cholesterol diet (5) Both plasma cholesterol and phospholipids increased the greatest effect being on the phospholipids resulting in a decreased C/P ratio The effect on C/P ratio is linearly related to the log dose and the lines for estrone and SC-6924 are parallel permitting relative activities to be calculated (Fig 5) Manvene (SC 6924) has 31% the lipid shifting effect of estrone

logically by determining the number of arteries involved and the degree of atherosclerosis

A qualitative comparison of the results is presented in Table VI and it should be noted that the data apply only to results obtained in cockerels Estradiol benzoate gives the expected results which are in

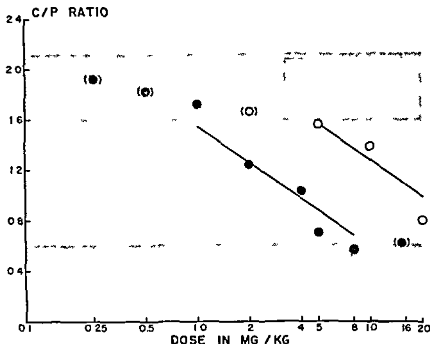


Fig 6 Effect of SC 6582 and estrone on reduction of the plasma cholesterol phospholipid ratio in cholesterol fed cockerels Closed circles = estrone open circles = SC 6582 Each point represents the mean of 4 to 28 birds Upper shaded area shows range of C/P ratio for untreated animals lower shaded area shows range of C/P ratio of animals fed a normal diet

agreement with the earlier report of Katz *et al* (13 18) SC 6924 and SC 8246 which are weak estrogens with no other significant endocrine properties have lipid effects similar to estradiol and also significantly prevent the occurrence of coronary lesions Testosterone propionate is without effect in cockerels SC 6582 which in addition to lipid effects has weak estrogenic androgenic and progestational effects does not prevent the occurrence of coronary lesions in cockerels It would appear then that when a given compound has a mixture of endocrine effects changes of the C/P ratio do not always predict the ultimate effect on the coronary lesions in the cockerel

SC 6582 was administered in doses of 5 10 20 and 40 mg per kg to the test cockerels, producing an effect similar to that of the previous compound SC 6924 (Fig 6) The lipid potency of SC 6582 is approximately 24% that of estrone (7)

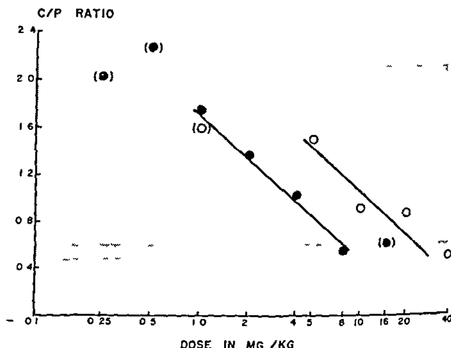


FIG 5 Effect of SC 6924 and estrone on reduction of the plasma cholesterol/phospholipid ratio in cholesterol fed cockerels Closed circles = estrone open circles = SC 6924 Each point represents the mean of 4 to 27 birds Regression lines fitted to data not in parentheses by method of least squares Upper shaded area shows range of C/P ratio for untreated animals lower shaded area shows range of C/P ratio of animals fed a normal diet

SC 8246 gave a dose response curve parallel to that of estrone (15) As judged by the change in C/P ratio SC 8246 has a lipid potency about equal to that of estrone (Fig 7)

Chronic Effects on Blood Lipids and Coronary Arteries Cook and Harris (8) have studied the effects of chronic administration of estradiol benzoate SC 6924 SC 6582 and SC 8246 in cockerels receiving an atherogenic diet The compounds were administered in oil subcutaneously each day for 8 to 10 weeks and determinations of blood lipids were made at intervals during the study At the end of the experiment atherosclerosis of the coronary arteries was estimated histo

teral ratio of 0 4 that is approximately 2½ times the subcutaneous dose must be administered orally in order to obtain the same degree of estrogenicity The compounds also produce the typical lipid shifts when administered orally to cockerels

TABLE VI
Comparative Chronic Effects of Steroids on Blood Lipids and Coronary Lesions in the Cockerel

	Estrogenicity	Androgenicity	Cholesterol	Phospholipid	C/P	Coronary lesions
Estradiol benzoate	++++	0	0	↑↑	↓	↓
SC 6924	+	0	0 or ↑	↑↑	↓	↓
SC 8246	+	0	0	↑↑	↓	↓
Testosterone propionate	0	++++	0	0	0	0
SC-6582	+	+	↓ or ↑	↑↑	↓	0

0 = No change
↑ Increase in concentration
↓ Decrease in concentration or incidence

Subacute Toxicity Studies An evaluation of subacute toxicity of SC 6582 SC-6924 and SC 8246 was made in rats receiving 50 mg of compound per kilogram of body weight intramuscularly each day for 2 weeks At this high dose level no untoward effects were observed other than those expected from known endocrine activities

Summary

The lipid/estrogenic ratio of standard estrogens is presented Other steroids have been studied and have been found to possess a higher lipid/estrogenic ratio than the standard estrogens The properties of those compounds are discussed

References

1 Adlersberg D *Am J Med* 23 769 (1957)
2 Arteriosclerosis—A Symposium *Minn Med* 38 731 829 (1955)
3 Barr D P *J Chronic Diseases* 1 63 (1955)
4 Biggers J D and Claringbold P J *J Endocrinol* 11 277 (1954)
5 Cook D L Calhoun D W and Edgren R A to be published
6 Cook D L Edgren R A and Harris T W *Circulation Research* 5 54 (1957)
7 Cook D L Edgren R A and Saunders F J *Endocrinology* 62 798 (1958)
8 Cook D L and Harris T W unpublished data
9 Drill V A and Riegel B *Recent Progr in Hormone Research* 14 29 (1958)
10 Edgren R A *Proc Soc Exptl Biol Med* 92 569 (1956)

Other Endocrine Effects SC 6924 SC 6582, and SC 8246 did not possess any cortisone like activity when tested for anti inflammatory effects eosinopenic effects, or neoglycogenetic activity They did not potentiate infections as do the standard corticoids These steroids are also without effect in causing sodium retention in acute tests in adrenal ectomized rats SC 6582 does not have progestational activity when

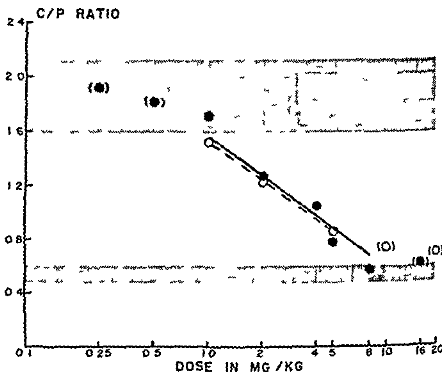


FIG 7 Effect of SC-8246 and estrone on reduction of the plasma cholesterol phospholipid ratio in cholesterol fed cockerels Closed circles and solid line = estrone open circles and broken line = SC-8246 Each point represents the mean of 4 to 28 birds Upper shaded area shows range of C/P ratio for untreated animals lower shaded area shows range of C/P ratio of animals fed a normal diet

injected directly into the uterine lumen of the spayed estrogen primed rabbit (McGinty technique) However some progestational activity was indicated following the administration of large parenteral doses in subacute toxicity tests

Oral Activity The oral/parenteral ratio for these compounds was determined by measuring estrogenic effects By such methods SC 6924 and SC-8246 are approximately as active following intragastric administration as they are when given parenterally SC 6582 has an oral/paren

in the aorta Such data might lead us to infer that some beneficial effects might be obtained in man With regard to SC 6582 this has a minimal estrogenicity and minimal androgenicity And all we can say is that although the C/P ratio fell in the cockerel we did not prevent the appearance of the lesions or alter the severity of the lesions I would judge therefore that as we work with other compounds we cannot base our conclusions on C/P ratios alone but will have also to examine the compounds over a 10-week period for the effects on the lesions in the cockerel

BOYLE I hate to muddy the water here any more but I would like to ask a question concerning the qualitative differences in lipoproteins in birds and in man On the blackboard I will roughly classify the cholesterol and phospholipid as it exists in humans we will say that this is an ultracentrifuge density gradient tube of 1 006 at the top and 1 21 at the bottom with 1 063 in the middle The S_f 17 and above are the very low density β lipoproteins and will float at this density 1 006 and they contain roughly about 1 1 cholesterol phospholipid If we take the beta lipoproteins which are the so-called beta 1 lipoproteins electrophoretically and have about a 1 035 density which will sink at 1 006 density but float at 1 063 density these are beta 1 or the S_f 0–15 of Gofman they will have roughly not quite 2 1 but closer to 1 5 to 1 cholesterol phospholipid ratio The only lipoprotein that has 0 5 cholesterol to phospholipid ratio is the α lipoprotein or the very high density lipoprotein which sinks at 1 063 Now in humans this lipoprotein is about 13 to 15% by dry weight of cholesterol and has anywhere from 22 to 35% phospholipid Therefore if we have an elevated cholesterol to phospholipid ratio in normal people we can assume that they have a greater abundance of these beta classes of lipoproteins in relation to the alpha lipoproteins There is only one incidence which Dr Furman mentioned in his paper in which you can have more phospholipid than cholesterol in humans in the absence of an increase in α lipoproteins and that is in primary biliary cirrhosis or in liver cell damage such as thorazine or methyltestosterone poisoning with jaundice If we take a bird (chicken) that say has 9 to 10% alpha lipoprotein levels normally he does not have a comparable cholesterol phospholipid ratio that would fit with any human distribution which you can conceive of Now as the cholesterol level goes up in the chickens with feeding of estrogens the phospholipid also disproportionately increases If this were true in humans the increase would have to be by definition in the α lipoproteins and yet the reports the other day show that this α lipoprotein drops to zero and the betas go way up in the chicken In the human this would give you a reversal of the cholesterol phospholipid ratio There are massive qualitative differences in these α and β lipoproteins in chickens and humans each having the reversal of cholesterol phospholipid ratios in each fraction The only way that I can see these estrogen induced changes so that the phospholipid can markedly increase with the cholesterol would be an increase in the α lipoproteins as in the case of normal humans or in liver damage I wonder if the liver functions were involved in this study on chicks Here the chicken on estrogen behaves like a human with primary biliary cirrhosis as far as lipoprotein distribution is concerned

DRILL We have not done any liver function studies in birds One of these materials SC 6924 was carried into dog chronic toxicity studies with determination of liver function tests such as alkaline phosphatase and serum bilirubin without producing any change I don t think you have to worry about the species difference when studying such compounds You do if you are studying the natural history

11 Edgren R A and Calhoun D W *Am J Physiol* **189** 355 (1957)
12 Huggins C and Jensen E V *J Exptl Med* **102** 347 (1955)
13 Katz L N and Stamler J Experimental Atherosclerosis C C Thomas Springfield Illinois 1953
14 Loeb H G *Proc Soc Exptl Biol Med* **49** 340 (1942)
15 Mueller G P Johns W F Cook D L and Edgren R A *J Am Chem Soc* **80** 1769 (1958)
16 Oliver M F and Boyd G S *Circulation* **13** 82 (1956)
17 Oliver M F and Boyd G S *Lancet* **2** 1273 (1956)
18 Pick R Stamler J Rodbard S and Katz L N *Circulation* **6** 276 (1952)
19 Robinson R W Higano N and Cohen W D *A M A Arch Internal Med* **100** 739 (1957)
20 Robinson R W Higano N Cohen W D Sniffen R C and Sherer J W Jr *Circulation* **14** 365 (1956)
21 Rubin B L Dorfman A S Black L and Dorfman R I *Endocrinology* **49** 429 (1951)
22 Russ E M Eder H A and Barr D P *Am J Med* **19** 4 (1955)
23 Sperry W M *Ind Eng Chem Anal Ed* **14** 88 (1942)
24 Steiner A Payson H and Kendall F E *Circulation* **11** 784 (1956)
25 Zlatkis A Zak B and Boyle A J *J Lab Clin Med* **41** 486 (1953)

Discussion

ROSENMAN *Dr Drill compound 6582 although weakly estrogenic* failed to protect the cholesterol fed cockerel against coronary atherogenesis despite a fall of serum cholesterol and of the C/P ratio similar to that induced by other estrogens found capable of inducing such protection May this therefore indicate that the protective effect of estrogens against cholesterol induced coronary atherogenesis in the cockerel is partly ascribable to effects of estrogens other than upon serum cholesterol or β lipoproteins? Although such effects are not now known they are again suggested by the fact that estrogens fail to protect the aortas of cholesterol *fed cockerels against induced atherogenesis despite their protective effect on* the coronary vasculature By analogy the protection of the premenopausal American female against clinical coronary disease may not entirely be ascribable to an estrogen induced level of serum β lipoproteins which is lower than her male counterpart only one of a great many attributes by which the sexes differ Nor does the presence of estrogen alone possibly account for the relatively low level of serum β lipoproteins in the American female as compared to American males since the rural Guatemalan female for example although not lacking in estrogen exhibits a relatively high level *of serum β-lipoproteins*

DRILL I agree with you that to obtain an effect on blood lipids does not mean necessarily that one will benefit the atherosclerotic process in man Our approach to the problem stems from the fact that estrone will produce what might be considered a beneficial change in blood lipids One could even argue this point but assuming such is the case we decided to look for a separation of lipid estrogenic properties within a given compound Such data have been presented today We have some indication from Dr Werthessen s work that estrogens or at least one *estrogen* can increase the rate of phospholipid synthesis in the calf aorta In our own laboratory Dr Ranney has completed some studies demonstrating that the naturally occurring estrogens can also increase the rate of phospholipid turnover

Pincus I would like to speak at this point We have made studies not with this compound in particular but with other compounds of this same structure that is having a 5 10 double bond which we obtained from you and particularly the 17-ethinyl compound and here we have noticed a very remarkable effect with administration of this material chronically to women (we have not studied men so far) It causes an increased free corticosteroid concentration in blood and a decrease in bound blood corticosteroid In excretion this is reflected in actual diminution of output of corticosteroids and the only analogy which we can assign to this in the various studies which have been done with steroids with what you might call the effects on blood and urinary corticosteroids is certain effects on the liver In other words there is a suggestion that this type of compound may have some effect on the liver in contrast to the others which we have also studied

Robinson We have been working with these compounds and we have found that the laboratory estimation of the lipid shifting effect of these materials has been borne out in man quite accurately and we will report on that subsequently in the use of Manvene However the laboratory estimation of estrogenicity has not been substantiated in man and we will discuss that further tomorrow There has been some dissociation we think in long term use of Manvene but it was in a small percentage of patients SC-6582 in laboratory animals was reported to have estrogenic effects on lipids with other characteristics of an androgen In man this material had an androgen effect on the lipids with a decrease in the α lipoprotein and increase in the β α lipoprotein cholesterol ratio The metabolic effects were anabolic with weight gain observed in most of the men SC-8246 the last compound, acted in man as an estrogen both in its lipid effects and in the clinical appearance of estrogenic side effects

Eder Dr Pick has described a considerable species difference between man and the chick. She pointed out that in the chick the administration of fairly large amounts of androgen in addition to estrogen resulted in no loss of the estrogen effect on the lipids In man the opposite effect is seen When a small dose of androgen is administered to a patient receiving fairly sizable doses of estrogen, the estrogen effect on the lipids is reversed but the feminizing effects persist. I believe *that this may account for some of the discrepancy in the prediction of the effect* of a substance which may have both androgenic and estrogenic activity

of the disease and want to apply the results to man Pharmacologically a related system can be picked and used to evaluate compounds which may be later studied in man provided a standard compound is available In other words (a) we know what estrone or ethinyl estradiol will do in man (b) we can determine what they will do in the cockerel (c) we can find other compounds that will act similarly in the cockerel and (d) we can predict what they will do in man Such compounds will not give the cockerel effect in man but will produce an estrone effect on blood lipids in man Pharmacologically you don t have to worry about the species cross over if you start with a standard material

BOYLE Estrogen has a specific effect of increasing the α lipoproteins and decreasing the β lipoproteins in humans with decrease in the cholesterol phospholipid ratio and just the converse of that occurs in the chicken

DRILL The two species are different but the compounds in either species produce effects characteristic for that species

WERTHESSEN It was that hermaphroditic compound of yours that changed the C/P ratio but had no effect on the plaques in the vessels wasn t it? Did this do anything in your *in vitro* test on the aorta?

DRILL We have not studied SC 6582 in such a way We will however follow your suggestion and try it

KATZ This is a rhetorical question directed to Dr Boyle Does it necessarily follow that under all circumstances the flotation of lipoproteins is associated with a constancy of its composition as regards cholesterol and phospholipids in man?

BOYLE For genetically normal human beings of both sexes it is constant and in various disease states such as liver damage nephrosis myxedema or what have you it can be qualitatively as well as quantitatively different I think this business of measuring lipoproteins in milligrams per cent quantitatively and ignoring the possible qualitative differences in these lipoproteins is a great oversight and should be gone into much more thoroughly as we are currently doing in our laboratory

PICK I want to say something to Dr Rosenman s criticism of the work I pointed out right in the beginning of my paper that the sex difference is only very marked in nations that subsist during the life span on what we consider a potentially atherogenic diet In all the nations which have been studied as to the occurrence of coronary artery disease and are found to have a low incidence the sex difference is very small and second I would like to ask Dr Drill one question In some conversations with Dr Cook and Dr Gantt from your laboratory I gathered that the compound 6582 with a low androgenic activity had a different lipid effect in humans It has an estrogenic lipid effect in the chicken lowering the C/P ratio and you showed it did not protect the coronary arteries but as I gathered from Dr Cook and I am asking you whether this is correct it had an androgenic effect on the blood lipids if given to man

DRILL Yes the finding of androgenic effects in man is based on Dr Eder s studies Is that correct, Dr Eder?

EDER Yes this compound appeared to have an androgenic effect on the lipoproteins in man

DRILL I have wondered if there was not some other effect of the molecule other than androgenicity that may be giving what one might term an antilipid effect The androgenicity of the compound is relatively low and perhaps so low that you would not have expected an androgenic effect on lipids Perhaps there is an antilipid metabolic effect inherent in the molecule that is not reflected in the androgenicity

CHAPTER 20

Effect of Adrenals Pituitary Liver, and Mucopolysaccharides on Blood Lipids

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In 1954 we reported that the delactescing action of heparin clearing factor was inhibited *in vitro* by the addition of plasma from animals administered cortisone exposed to cold or subjected to the nephrotic syndrome (18) The data are presented in Table I Clearing factor (CF) was from rats administered 4 mg heparin/kg intravenously Substrate was plasma from alimentary hyperlipemic dogs The column LP shows the opacity of the lipemic plasma (LP) LP + CF shows that

TABLE I

INHIBITOR OF LIPEMIA CLEARING EFFECT OF CF AND CF + CFI RAT PLASMA ON LIPEMIC DOG PLASMA (LP)

No rats CF[b]	Source of CF[b]	No rats CFI	Source of CFI	Average % light transmission LP	LP + CF[b]	LP + CF[b] + CFI
6	Heparin	15	Cortisone	9	96	10
6	PDHA	6	Cortisone	22	86	22
6	DCA	6	Corti one	20	97	21
6	Heparin	6	Stress	20	96	21
6	Heparin	4	0 1 AKS	18	86	41
6	Heparin	3	0 2 AKS	23	86	27
6	Heparin	5	0 4–0 8 AKS	21	86	23

Milliliter antikidney serum/100 g of rat.
[b] CF = Lipemia clearing factor
CFI = Lipemia clearing factor inhibitor

the CF was actively delactescent The last column shows that plasma from cortisonized rats rats exposed to cold or those receiving anti kidney serum when added to an active clearing system prevented the usual increase in light transmission In other experiments we established the presence of an *in vitro* clearing factor inhibitor in the plasma obtained from humans centrifuged at 12 g human nephrotics familial hyperlipemics surgical patients and obstetrical patients (23) Several other laboratories have since reported a similar inhibitor in the following conditions familial hyperlipemia in humans (12) administration of cortisone (7) administration of toxic doses of alloxan (14) and fre

quent bleeding of rabbits (16) The occurrence of a dialyzable inhibitor has now also been reported to be present in normal bovine plasma (13) Chalmers *et al* (24) have isolated from the urine of humans a similar peptide with lipid mobilizing properties

Injection of CFI (clearing factor inhibitor) into a variety of species including humans resulted in hyperlipemia As will be shown later the hyperlipemia did not depend on clearing factor inhibition in vivo but was due to lipid mobilization from the depots We have therefore dropped the term CFI in favor of lipid mobilizer (LM) The data are shown in Table II

Since LM was first demonstrated in rats receiving cortisone or exposed to stress we determined the role of the adrenals in releasing LM *Bacterial pyrogens or convulsions were used as stressors in adrenalectomized* or hypophysectomized rats Table III shows that a pyrogenic reaction produced hyperlipemia in intact rats but not in adrenalectomized or hypophysectomized ones although fever was present in all groups

TABLE III

HYPERLIPEMIA BY 50 μ PIROMEN/KG I V IN RATS (2 HOUR P INJECTION)

	Plasma lipid levels (mg %)		
	Cholesterol	Fatty acids	Lipid P
Intact	180	210	10
Adrenalectomized	72	108	6
Hypophysectomized	70	115	6

Table IV shows that diisopropylfluorophosphate (DFP) produced hyperlipemia in intact rats but not in those adrenalectomized or hypophysectomized although convulsions occurred in all groups Nonconvulsant doses of DFP did not produce hyperlipemia in intact rats DFP was originally used because Shore and his colleagues had shown that it was a potent inhibitor of lipoprotein lipase (20) The subconvulsant doses we employed were calculated to give plasma concentrations considerably in excess of those found to inhibit lipoprotein lipase *in vitro* The results of this experiment made it doubtful that LM caused hyper

TABLE IV

HYPERLIPEMIA BY 1 MG DFP/KG I V IN RATS (2 HOUR P INJECTION)

	Plasma lipid levels (mg %)		
	Cholesterol	Fatty acids	Lipid P
Intact	190	260	11
Adrenalectomized	62	110	5
Hypophysectomized	58	110	7

TABLE II

MEAN CONCENTRATION (MG %) OF LIPIDS IN PLASMA OF ANIMALS 1 HOUR AFTER INJECTION OF LM

Animal	No	mg/kg	Total Ch[a]		FA[b]		LP[c]		OD[d]	
			Initial	1 hr	Initial	1 hr	Initial	1 hr	Initial	1 hr
Mice	100	0.5	53	174	148	450	5	12	0.05	0.90
Rats	475	0.10	72	190	155	480	5	13	0.04	0.86
Guinea pigs	100	0.50	66	185	160	140	5	12	0.06	0.80
Rabbits	10	0.50	50	172	200	420	5	11	0.05	0.86
Dogs	20	1.0	86	168	209	430	6	11	0.05	0.92

[a] Ch = cholesterol [b] FA = fatty acids [c] LP = lipid phosphorus [d] OD = optical density

TABLE V

EFFECT 1 MG DIALYZATE OF HOG POSTERIOR PITUITARY LOBES/KG IV ON PLASMA LIPID COMPONENTS OF VARIOUS SPECIES

	Time in hours post injection																	
	0			1			2			4			6			24		
Species	CH	FA[b]	LP	CH	FA	LP	CH	FA	LP	CH	FA	LP	CH	FA	LP	CH	FA	LP
Intact rats (10/hr)	62	104	5	196	212	9	262	303	10	302	353	12	348	392	12	184	212	8
Hypophysectomized rats (20/hr)	58	92	5				242	303	11									
Adrenalectomized rats (20/hr)	64	88	5				258	312	11									
Mice (10/hr)	52	95	5	195	208	8	312	340	12	402	386	15	440	490	10	120	212	8
Guinea pigs (10/hr)	85	102	5	186	195	9	245	260	10	322	330	11	385	402	13	84	92	6
Rabbits (12/sample)	62	90	5	190	262	9	240	304	11	320	386	13	360	412	14	75	142	8
Dogs (6/sample)	1[illegible]0	160	6	290	340	11	380	400	18	412	400	19	402	440	18	196	220	9

CH = Total plasma cholesterol

[b] FA = Total fatty acids

[c] LP = Lipid phosphorus

All values in mg %

lipemia by *in vivo* neutralization of lipoprotein lipase They also indicate that *in vitro* effects of inhibitors cannot be transferred to *in vivo* phenomena The findings suggested that the hyperlipemia was of more complex origin and that it might be another manifestation of the general adaptation syndrome via the anterior pituitary and adrenal cortex Testing this hypothesis resulted in the surprising observation that injection of cortisone into hypophysectomized rats failed to release LM We then had to consider the possibility that the posterior pituitary played a role in the release of LM Table V shows the lipemia inducing properties of dialyzate from the posterior pituitary of hogs No such activity was demonstrable for Pitocin Pitressin extracts of anterior lobe or skeletal muscle Inhibitory action of the adrenals and pituitary on the heparin clearing action has recently been reported by Pinter Kovacs and Karady (15) Hypophysectomy has been reported by Heymann and Haekel (11) and by Bally and Neema (3) to either abolish or largely prevent the hyperlipemia of experimental nephrosis

The experience with DFP suggested investigation of the mechanism by which three other hyperlipemic agents act Two of these protamine (5) and toluidine blue (8) are considered to be specific antagonists of heparin and they are supposed to produce hyperlipemia by inactivating the heparin moiety of lipoprotein lipase The third hyperlipemic agent is phenylhydrazine (4 17) Table VI shows that protamine hyperlipemia depends upon the presence of the adrenals and pituitary In this respect it resembles other stressor or toxic agents Were it acting peripherally on tissue lipoprotein lipase protamine would be expected to produce hyperlipemia in the absence of these endocrines Shotz and Page (21) have recently noted identical results obtained in a similar experiment with protamine in hypophysectomized and adrenalectomized rats We have found that the toluidine blue hyperlipemia occurs only after repeated administration of the dye and that it is not due to antiheparin action but to anemia and hepatotoxicity Phenylhydrazine is not a heparin antagonist and produces lipemia by a mechanism similar to that of toluidine blue In our experience substances which act as stressors and which at the same time are hepatotoxic can produce lipemia in the animal to whom they are administered Stressors without hepatotoxic potential release LM whose lipemic action can be demonstrated in animals sensitized by hepatotoxins

LM differs from the other agents discussed in that it produces hyperlipemia in the absence of either the adrenals or pituitary as can be seen in Table V These data suggest that the hyperlipemia action of LM may be due to a direct neutralization of clearing factor either in the circulation or in the tissues As has already been mentioned consider

TABLE VII
ROLE OF LIVER ON FAT MOBILIZATION IN DOGS

		Time (hr) $\overline{P}$ Injection of LM											
		0			1/4			1			2		
		Plasma lipids (mg %)											
		CH	FA[b]	LP[c]	CH	FA	LP	CH	FA	LP	CH	FA	LP
Fasted dogs with normal liver	Superior mesenteric vein (prehepatic)	60	140	4	62	310	4	58	325	5	58	350	5
	Posthepatic vena cava	82	148	6	92	160	6	100	150	5	100	152	5
Fasted dogs with damaged liver	Superior mesenteric vein (prehepatic)	52	130	3	56	325	3	70	400	6	75	400	6
	Posthepatic vena cava	82	150	6	90	162	6	175	300	6	300	325	9

CH = Total cholesterol
[b] FA = Total fatty acids
LP = Lipid phosphorus

able doubt is cast on this view by the fact that DFP does not induce hyperlipemia in concentrations that abolish the lipolytic activity of lipoprotein lipase More direct studies suggest that LM mobilizes triglycerides and that the liver determines whether this will be manifested as peripheral hyperlipemia

TABLE VI

HYPERLIPEMIA BY 10 MG PROTAMINE SO_4/KG I V IN RATS (1 HOUR P INJECTION)

	Plasma lipid levels (mg %)		
	Cholesterol	Fatty acids	Lipid P
Intact	220	275	12
Adrenalectomized	72	130	6
Hypophysectomized	70	130	6

The role of the liver on the consequences of mobilization of fat by LM in rats has been described in detail (19) Animals sacrificed at 0 hour were controls which had not received LM The liver at this time had lipid values considerably higher than those reported in the literature for the Wistar strain and were considered to be fatty although they were not so designated by microscopic examination The plasma values were within the normal range Fifteen minutes after injection of LM there was a significant increase in the total fatty acids of the liver which was not associated with significant changes in either the cholesterol or lipid phosphorus nor with changes in any of the lipid components of the plasma One hour following injection the total fatty acid content of the liver had returned to preinjection level and was associated with significant lowering of cholesterol and lipid phosphorus All lipid values in the plasma were elevated The liver lipid values remained depressed for the next 3 hours and during this time the hyperlipemia was intense As the hyperlipemia subsided the values for the liver lipids increased, particularly those for total fatty acids The data suggest that LM promptly mobilized neutral fat to the liver The latter did not filter this but added cholesterol and phospholipid and peripheral hyperlipemia resulted The data do not indicate whether the mobilized fat was utilized

The role of the liver in lipemia by LM in dogs is shown in Table VII Samples of blood taken from the superior mesenteric vein and post hepatic inferior vena cava of fasted dogs with no signs of impaired liver function showed an elevation of neutral fats in the blood brought to the liver following injection of LM The portal hypertriglyceridemia was not manifested as peripheral hyperlipemia This is in contrast to dogs that had been exposed to Chlordan In these LM also induced portal

a slight arteriovenous difference of cholesterol suggestive of a possible pathway for cholesterol excretion through the intestines None of the patients preoperatively had LM in any of the samples as measured by *in vitro* inhibition of delactescence Following the surgical procedure all specimens contained such activity The data are presented in Table IX.

The liver is not the only organ capable of removing large quantity of lipid It is well established that hyperlipemia occurs in pregnant females but not in the new born The cholesterol and total fatty acids of maternal venous blood and umbilical cord blood in humans shown in Table X are in agreement with published data More direct measurements obtained by simultaneously sampling maternal blood from the cubital vein uterine sinus umbilical cord and fetal femoral vein blood are shown in Table XI Maternal cord and fetal blood contained LM

TABLE X

PLASMA LIPID LEVELS (MG %) AT DELIVERY

Maternal (CV)		Cord		Baby	
CH[b]	FA[c]	CH	FA	CH	FA
242	260	90	196	90	166
307	348	79	99		
286	342	82	109		
287	311	95	112		
274	296	85	98		
303	358	72	88		
288	302	81	99		
272	303	84	96		
292	334	76	91		
282	344	84	99	78	104
244	380	90	104		
197	312	71	86		
236	374	99	130		
199	426	76	97	82	91
197	288	71	90	73	104
328	628	103	158	102	174
248	292	87	112	71	86
199	212	84	11[illegible]	82	91
[illegible]92	318	102	114	100	112
290	341	82	91	71	103
[illegible]0[illegible]	358	78	96	80	93
308	399	82	93	88	99
212	296	78	103	81	92

CV = Anticubital vein
[b] CH = Total cholesterol
FA = Total fatty acid

hypertriglyceridemia this was manifested as peripheral hyperlipemia to which the liver had apparently added cholesterol and phospholipid.

It has already been stated that a variety of stresses release LM into the circulation and evidence for similar hormonal control in humans is presented in Tables VIII and IX. Table VIII shows that surgical procedures result in a marked elevation of total fatty acids in the portal

TABLE VIII

ARTERIOVENOUS DIFFERENCE IN PLASMA LIPIDS IN OMENTAL CIRCULATION (MG %)

	Preoperative						Postoperative					
	Cholesterol			Total fatty acids			Cholesterol			Total fatty acids		
Case	A	V	A/V%	A	V	A/V%	A	V	A/V%	A	V	A/V%
1	228	212	93	280	284	100	280	202	73	308	499	160
2	238	202	85	252	268	83	290	284	98	326	852	262
3	252	228	90	274	286	104	251	220	88	290	446	154
4	226	202	90	312	314	100		226			413	
5	232	201	86	338	312	93	248	209	84	360	440	122
6	312	302	96	348	326	94	346	338	97	402	648	162
7	186	154	83	214	218	100	196	184	94	232	386	166
8	186	142	76	214	208	98	196	188	96	394	786	199

TABLE IX

EFFECT OF SURGICAL STRESS ON CLEARING OF LIPEMIC DOG PLASMA *in vitro* BY HEPARIN CLEARING FACTOR PLASMA

	Change in optical density				
	Time in hours				
	0	1/4	1/2	3/4	1
LP + HCF	0.70	0.49	0.31	0.28	0.28
LP + HCF + LM	0.75	0.73	0.72	0.72	0.72
LP + HCF + Prehep (*start of op*)	0.69	0.50	0.28	0.26	0.26
LP + HCF + Posthep (start of op)	0.69	0.51	0.31	0.30	0.30
LP + HCF + Prehep (end of op)	0.88	0.80	0.79	0.78	0.78
LP + HCF + Posthep (end of op)	0.78	0.75	0.74	0.74	0.74

circulation. This was not manifested as a significant hyperlipemia in the peripheral circulation. The portal hypertriglyceridemia was sometimes evidenced visually from the marked lactescence of the plasma. The samples were from the gastroepiploic vein and gastroepiploic artery. The arterial blood is representative of peripheral or posthepatic circulation and the venous blood is representative of portal or prehepatic circulation. In other experiments the samples of peripheral circulation were obtained from the cubital vein and the results in these experiments were no different from those shown in Table VIII. Table VIII also reveals

a slight arteriovenous difference of cholesterol suggestive of a possible pathway for cholesterol excretion through the intestines None of the patients preoperatively had LM in any of the samples as measured by *in vitro* inhibition of delactescence Following the surgical procedure all specimens contained such activity The data are presented in Table IX

The liver is not the only organ capable of removing large quantity of lipid It is well established that hyperlipemia occurs in pregnant females but not in the new born The cholesterol and total fatty acids of maternal venous blood and umbilical cord blood in humans shown in Table X are in agreement with published data More direct measurements obtained by simultaneously sampling maternal blood from the cubital vein uterine sinus umbilical cord and fetal femoral vein blood are shown in Table XI Maternal cord and fetal blood contained LM

TABLE X
PLASMA LIPID LEVELS (MG %) AT DELIVERY

Maternal (CV)[a]		Cord		Baby	
CH[b]	FA[c]	CH	FA	CH	FA
242	260	90	196	90	166
307	348	79	99		
286	342	82	109		
287	311	95	112		
274	296	85	98		
303	358	72	88		
288	302	81	99		
272	303	84	96		
292	334	76	91		
282	344	84	99	78	104
244	380	90	104		
197	312	71	86		
236	374	99	130		
199	426	76	97	82	91
197	288	71	90	73	104
328	628	103	158	102	174
248	292	87	112	71	86
199	212	84	112	82	91
292	318	102	114	100	112
290	341	82	91	71	103
302	358	78	96	80	93
308	399	82	93	88	99
212	296	78	103	81	92

[a] CV = Anticubital vein
[b] CH = Total cholesterol
[c] FA = Total fatty acid

and Table XII shows the *in vitro* clearing inhibition It is interesting to note that the fetal circulation contained LM The human new born does not have mesenteric or omental fat depots Either these have not yet been deposited or they have been depleted by LM

TABLE XI

PLASMA LIPID LEVELS (MG %) DURING CAESAREAN SECTION

Maternal											
RA[a]		CV[b]		US[c]		FV[d]		Cord		Baby	
CH	FA	CH	FA	CH	FA	CH	FA	CH	FA	CH	FA
291	346	282	408	280	392			81	92		
288	342	278	314	288	342			78	84		
		394	395	400	457	400	457	109	121	92	103
360	490	356	484	382	501			99	113	92	110

[a] RA = Radial artery
[b] CV = Anticubital vein
US = Uterine sinus
[d] FV = Femoral vein

TABLE XII

EFFECT OF MATERNAL CORD AND FETAL BLOOD ON LIPEMIA CLEARING *in Vitro*

	Change in optical density				
	Time in hours				
	0	1/4	1/2	3/4	1
LP + HCF	0 72	0 41	0 28	0 20	0 18
LP + HCF + LM	0 77	0 76	0 75	0 75	0 75
LP + HCF + Cord	0 73	0 64	0 63	0 63	0 63
LP + HCF + Maternal cubital vein	0 73	0 68	0 65	0 64	0 64
LP + HCF + Uterine sinus	0 75	0 69	0 68	0 68	0 66
LP + HCF + Maternal femoral artery	0 75	0 74	0 74	0 74	0 72
LP + HCF + Baby femoral vein	0 76	0 75	0 74	0 73	0 72

The data presented thus far appear to establish an endocrine control of lipid mobilization The adrenal cortex the anterior pituitary and the posterior pituitary are involved The pathway appears to be a common one for immediate mobilization of lipids during a catabolic process The sequence is anterior pituitary adrenal cortex posterior pituitary release of LM The target for LM is the mesenteric fat depot which responds with increased permeability and liberation of triglyceride into the portal circulation

In previous studies we demonstrated that hyaluronidase partially depolymerized hyaluronic acid and deoxycorticosterone had opposite effects to those of cortisone on permeability of the ground substance

These effects are largely due to alteration of polymerization of the mucopolysaccharides in the ground substance It became of interest therefore to determine whether lipid mobilization could be prevented or antagonized by endocrines and other agents that affect the ground substance

The effect of hyaluronidase on the permeability of blood vessels and tissues to circulating lipids of hyperlipemic animals is shown in Table XIII During the first 5 weeks of treatment, the hyaluronidase treated rabbits had consistently lower blood cholesterols than those administered cholesterol without hyaluronidase After the fifth week there was an escape from the hypocholesterolemic action The reasons for this are not clear but coincidentally there was a marked elevation of antihyaluronidase titer in the blood of the hyaluronidase treated rabbits The atheromas in the aorta were at least as severe in the latter group as in those receiving only cholesterol In some instances they were considerably more severe There was also a marked deposition of fat in the liver kidneys and spleen of the hyaluronidase treated rabbits These findings indicate that hyaluronidase increased the permeability of various tissues for lipid A similar effect has also been reported by Cali (6) Enhancing effects of hyaluronidase have also been reported by Wong *et al* (22) in rabbits rendered excessively hyperlipemic by combined administration of cortisone and cholesterol Hyaluronidase lowered the blood lipids but enhanced atheromatous lesions and resulted in marked deposition of lipids in the liver Adlersberg *et al* (1) and Dury and DiLuzio (10) have reported that although cortisone elevates hyperlipemia in rabbits it does not enhance the formation of atheromas This might be due to the antipermeability effect of cortisone

The effect of hyaluronidase on plasma lipids suggested that a lipid clearing factor was involved We demonstrated that plasma from rats administered hyaluronidase partially depolymerized hyaluronate or deoxycorticosterone caused delactescence when added to lipemic plasma The clearing was not associated with lipolysis and therefore differed from heparin clearing factor (2) see Table I Table XIV shows the effects of deoxycorticosterone acetate (DCA) on LM hyperlipemia in rats DCA was injected into intact, adrenalectomized and hypophysectomized rats Two hours later the animals were bled and the plasmas separated. The plasma from the intact rats was coded I that from adrenalectomized ones as A and that from hypophysectomized ones as H Another group of rats received injections of LM One hour later 25% of the rats were administered either I A or H intravenously The same experimental design was employed for adrenalectomized and

TABLE XIII

EFFECT OF HYALURONIDASE ON TOTAL BLOOD CHOLESTEROL LEVELS AND AORTIC LESIONS OF RABBITS ON AN ATHEROGENIC REGIMEN

Treatment	Total blood cholesterol (mg %) Weeks of experiment								Degree of atheromatosis of aorta	
	0	1	2	3	4	5	6	7	Thoracic	Abdominal
Control	82	81	70	95	93	89	98	75	0	0
5% Cottonseed oil	70	81	87	98	84	85	103	88	0	0
5% Cottonseed oil + 0 5% cholesterol	96	303	155	178	209	241	496	377	1 6	1 8
5% Cottonseed oil + 1000 TRU hyaluronidase/kg S C /day	72	86	96	93	76	76	97	101	0 5	0 2
5% Cottonseed oil + 0 5% cholesterol + 1000 TRU hyaluronidase/kg S C /day	72	144	96	114	121	141	267	641	2 1	2 6

hypophysectomized rats One hour after the injection of DCA plasma the rats were anesthetized and plasma obtained for lipid analysis It will be seen that DCA plasma had no effect on the blood lipids of untreated rats but that DCA plasma abolished the hyperlipemic action of LM The DCA plasma was effective whether obtained from intact adrenalectomized or hypophysectomized rats It may be assumed that the antagonistic effect was peripheral and probably at the site where LM acts The possibility that this effect is due either to retention of the mobilized lipids by the liver or to demobilization by altering permeability of the fat depots is being investigated An effect of DCA on blood lipids in dogs has been reported by DiLuzio *et al* (9)

TABLE XIV
EFFECT OF DCA ON LM HYPERLIPEMIA IN RATS

	Intact		Adrex		Hypex	
Treatment	CH	FA	CH	FA	CH	FA
None	78	100	79	92	72	90
LM	199	304	189	297	190	340
LM + I[a]	78	92	71	85	73	92
LM + A[b]	81	99	79	93	71	99
LM + H	80	90	85	91	70	100

I = Plasma from intact rats treated with DCA
[b] A = Plasma from adrenalectomized rats treated with DCA
[c] H = Plasma from hypophysectomized rats treated with DCA

Since the effect of DCA in hyperlipemic rats resembled that of hyaluronidase in hyperlipemic rabbits it was of interest to investigate the effect of partially depolymerized hyaluronic acid (PDHA) on the hyperlipemia induced by LM or by plasma from patients with familial hyperlipemia (AA and DW) The hypocholesterolemic action of PDHA in nephrotic rats has already been reported (25) The data are presented in Table XV Injection of LM or plasma from hyperlipemic patients produced hyperlipemia in rats in 1 hour Injection of 5 mg of PDHA/kg

TABLE XV
EFFECT OF PDHA ON HYPERLIPEMIA IN RATS

Treatment	FA	CH
LM	326	272
AA	299	246
DW	306	285
LM + Normal human plasma	338	280
LM + PDHA	94	82
AA + PDHA	103	96
DW + PDHA	92	85

intravenously 1 hour later restored the blood lipids to normal values The effect of PDHA was similar to that of DCA and hyaluronidase PDHA is a nonsulfated mucopolysaccharide Similar nonsulfated partially depolymerized mucopolysaccharides are probably activated by administration of hyaluronidase and DCA It has already been mentioned that these agents release a delactescing factor which differs from lipoprotein lipase in not exhibiting lipolytic activity We have reported similar delactescing properties for other nonsulfated mucopolysaccharides (2) The antihyperlipemic and delactescing properties of nonsulfated mucopolysaccharides differ from the effects of sulfated mucopolysaccharides such as heparin The latter release lipoprotein lipase which is not always associated with delactescence or hypolipemic action The most striking instance of this is the failure of heparin to affect the lactescence or degree of lipemia in rabbits subject to repeated bleeding (16) or in rats injected with LM

Summary

1 An endocrine control of lipid mobilization is demonstrated. Cortisone stress and various clinical states release a lipid mobilizing substance (LM) from the posterior pituitary

2 LM releases triglycerides from the mesenteric fat depots to the liver

3 Peripheral hyperlipemia results when the liver is unable to utilize the triglyceride load The liver contributes the cholesterol and lipid phosphorus of the hyperlipemia

4 LM hyperlipemia is antagonized by partially depolymerized nonsulfated mucopolysaccharides but not by heparin

References

1 Adlersberg D Schaefer L E and Wang C I *Science* **120** 319 (1954)
2 Baeder D H Shore B Gofman J W and Seifter J *Federation Proc* **15** 396 (1956)
3 Bally P and Neema S *J Clin Invest* **36** 872 (1957)
4 Boggs T and Morris R S *J Exptl Med* **111** 553 (1909)
5 Bragdon J H Havel R J and Gordon R S Jr *Am J Physiol* **189** 63 (1957)
6 Cali A *Riv anat patol e oncol* **5** 1 (1952)
7 Constantinides P and Canes A *Federation Proc* **14** 31 (1955)
8 Day A J Wilkenson G K Schwartz C J and Peter J A *Australian J Exptl Biol Med Sci* **35** 277 (1957)
9 DiLuzio N R Shore M L and Zilversmit D B *Metabolism Clin and Exptl* **3** 424 (1954)
10 Dury A and DiLuzio N R *Am J Physiol* **187** 45 (1955)
11 Heyman W and Haekel D B *Metabolism Clin and Exptl* **6** 169 (1957)
12 Klein E and Lever W F *Proc Soc Exptl Biol and Med* **95** 565 (1957)

13 Lever W F and Klein E *J Invest Dermatol* **29** 465 (1957)
14 Meng H C and Hadley J I *Abstr 20th Intern Physiol Congr* p 637 (1956)
15 Pinter I Kovacks J and Karady S *Acta Physiol Acad Sci (suppl)* **11** 77 (1957)
16 Robinson D S and Harris P M *Biochem J* **66** 18P (1957)
17 Rosenthal F and Meer K *Arch exptl pathol u Pharmakol Naunyn Schmiedebergs* **91** 261 (1921)
18 Seifter J and Baeder D H *Proc Soc Exptl Biol Med* **87** 276 (1954)
19 Seifter J and Baeder D H *Proc Soc Exptl Biol Med* **95** 747 (1957)
20 Shore B Nichols A V and Freeman N K *Proc Soc Exptl Biol Med* **83** 216 (1953)
21 Shotz M C and Page I H *Circulation* **16** 515 (1957)
22 Wang C I Shaefer L E and Adlersberg D *Circulation Research* **3** 293 (1955)
23 Zarafonetis C J D Seifter J Baeder D H and Kalas J *Abstr J Lab Clin Med* **50** 965 (1957)
24 Chalmers T M Kekwick A and Pawan G L S *Lancet* **i** 866 (1958)
25 Seifter J Baeder D H Beckfield W J Sharma G P and Ehrich W E *Proc Soc Exptl Biol and Med* **83** 468 (1953)

Discussion

White What effects do the mucopolysaccharides have in your studies?

Seifter This depends on two things (*1*) the nature of the mucopolysaccharide (2) the chemical purity I can only illustrate this by a recent experience You will notice that we have studied a variety of mucopolysaccharides The mucopoly saccharide heparitin sulfate is excreted in the urine of humans suffering with Hurler s syndrome and was of interest to us because it is not anticoagulant and was found to release clearing factor Heparitin sulfate is a well characterized com pound so Dr Karl Meyer gave us some prepared from bovine lung It was in jected over a period of 6 weeks to weanling male mice and produced an effect we have not seen with other mucopolysaccharides It caused a deposition of fat in the gonad and suprascapular area Thes fat depots are not readily mobilized This experiment will now have to be reproduced with a known sample obtained from the urine of gargoyles A similar situation and more to the point exists when two authentic samples of chondroitin sulfate B have given us different results in the clearing reaction Some of the other mucopolysaccharides such as hyaluronic acid isolated from a Group A *Streptococcus pyogenes* injected over a long period of time have not done anything

White Do your polysaccharides have any effect on the activity of your clear ing factor inhibitor either *in vitro* or *in vivo*?

Seifter Nonsulfated mucopolysaccharides release *in vivo* a clearing factor which is not a lipase Some mucopolysaccharides reverse the lipemia induced by CFI (LM)

Werthessen I would like to ask three questions First what is the present status of your lipid mobilizing factor as a posterior pituitary hormone? Second how long have you administered it now in experimental animals without obtaining atheroma? And finally have you used it as a therapeutic agent in animals in which atherosclerosis had been developed?

SEIFTER As far as we can determine it is from the posterior pituitary We have isolated LM from the posterior pituitary of hogs which have not been stimulated in any way Administration of LM for the production of atheroma has been the biggest disappointment We have administered LM over a period of 3 months which I think is adequate for this sort of experiment We had cholesterol levels in the blood identical with those that were obtained with feeding cholesterol The rabbits fed cholesterol had atheroma while the LM treated rabbits did not For a while we thought LM might be producing a medial sclerosis which it is not Administering LM along with cholesterol in the diet may result in somewhat more severe lesions but this is subject to argument So far as we can see hyperlipemia by LM does not play a role in producing atheromas in rabbits

WERTHESSEN What about animals in which atheroma had been induced and then treated with the agent?

SEIFTER We haven t been able to demonstrate any prophylactic effect or regression effect So far as we can see LM does not influence the course of experimental atherosclerosis in rabbits

FURMAN Albrink and Man at Yale (*Am J Digest Diseases* 2 649 1957) have shown that the feeding or infusion of glucose or other maneuvers leading to increased glucose utilization will decrease postprandial lipemia What does glucose feeding or infusion do to the lipemia resulting from the administration of CFI?

SEIFTER It is similar to a protamine lipemia If you administer glucose to a fasted animal neither protamine nor LM produce hyperlipemia Hyperlipemia induced by either protamine or LM is not abolished by administration of glucose

HOLMAN There must be questions in Dr Seifter s mind that elude me to some extent He has a variety of experimental observations here I don t think he has put all his cards on the table here for some reason In the first place he has evidence of cord blood versus maternal blood I think that might well be explained on the basis of epithelial covering of the villi I think it would reflect active work on the part of the epithelium to maintain the differential between maternal and fetal blood He has presented a variety of evidence having to do with operative procedures and this intrigues me no end of being able to find the difference in arteriovenous blood from the omentum following operative procedures It is a complicated proposition that could conceivably depend upon trauma or upon venous or lymphatic drainage The question I would like to ask Dr Seifter with regard to operative procedures in particular is whether he believes that something liberated by the posterior pituitary could be localized and so explain arteriovenous differences in the fat from the omentum or the mesenteric depot I don t know whether that is a fair question or not More specifically I would like to ask what prompted you to determine the difference between arteriovenous blood following laparatomy?

SEIFTER I hope we have laid our cards on the table I have nothing to hide We don t know in what manner the placenta acts as a barrier to the passage of lipids Now you might raise the question that the fetus has the inhibitor in its plasma and yet is not hyperlipemic This has puzzled us too but pathologists tell me that the fetus has practically no mesenteric fat nor does it have omental fat We sought to demonstrate in patients undergoing surgery that the trauma or stress of the procedure released CFI which mobilized lipids to the liver The liver determined whether or not there was a peripheral hyperlipemia

FREEDBERG Can you tell us what the blood supply is to the omentum? Is it the same as the mesenteric system? It seems to me there are anastomoses

SEIFTER The samples were taken from the gastroepiploic vein and artery The arterial sample would be representative of the systemic circulation The venous sample would be a fair representation of the portal circulation because the right gastroepiploic drains into the superior mesenteric vein and the left gastroepiploic drains into the splenic vein

FREEDBERG But it also drains into the systemic system does it not? That s the question I m asking

SEIFTER The communication between the portal and general venous system occurs through the superior hemorrhoidal branches of the inferior mesenteric vein which communicate with the middle hemorrhoidal branches of the internal iliac

POPJÁK I would like to make some comments about the observations on lipid content of the fetal and maternal blood and on the action of the placenta as a barrier between the two The fetal lipids are derived within the fetus almost exclusively by synthesis within the fetal tissues The barrier between the maternal and the fetal circulation as regards lipids is quite extraordinary The placenta has a synthetic ability of its own but in addition to that it has a great avidity for maternal plasma lipids Certainly in animals with a hemochorial or hemoendothelial type of placenta there is a good deal of lipid absorption by the endothelial cells of the fetal placenta but none of the absorbed lipid is transmitted to the body of the fetus

SEIFTER We recognize that the fetal lipids were probably produced within the fetus itself What we attempted to illustrate was that the maternal hyperlipemia was not manifested in the fetus in spite of the fact that both maternal and fetal blood contained CFI

DOUGHERTY I think that one of the points that Dr Seifter made is that these substances which he used were heparin binders that is Pitressin toluidine blue protamine etc I have pointed out that the work of Dr Berliner and myself demonstrated that ACTH brought about an enhanced amount of both blood cholesterol and cholesterol deposited in the lung We have heard other papers that indicate the same thing I would like to go on to a second order type of thinking and that is to point out that Dr Higginbotham and I indicated some time ago that ACTH is one of the most potent binders of heparin Consequently with a stress phenomenon it is possible that with the outpouring of ACTH or its release from peripheral binding sites it could inactivate heparin or that these substances such as toluidine blue Pitressin etc which are hyperlipemic inhibit the binding of ACTH with heparin therefore causing an increased amount of circulating ACTH and causing hyperlipemia I think that if we combine these two studies we can see quite clearly that ACTH could be a hyperlipemic factor and that if we inhibit its peripheral binding sites on these polysaccharides we could produce hyperlipemia

CHAPTER 21

The Possible Relationship of the Emotions to Clinical Coronary Heart Disease

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The recent preoccupation with lipids and especially dietary lipids in the pathogenesis of coronary artery disease has induced such intensity of thought that the mere mention of other possible causal factors occasionally evokes rather remarkable denials (4 48 69) albeit lack of scientific negation The present conference implies of course that the endocrine system may play an important pathogenetic role in atherogenesis and clinical coronary heart disease The proposition that those forms of socioeconomic stress unique to Western societies may play a fundamental causal role in the increasing incidence of clinical coronary disease in industrialized groups as well as in the latter s higher incidence of coronary disease morbidity and mortality relative to natives and groups of nonindustrialized societies (31–33 60) so strongly implicates *the hypothalamic pituitary adrenal axis that its inclusion is justified in* the present discussion Although the evidence in support of this belief cannot be extensively reviewed in the time allotted to this presentation certain phases of this problem are worthy of emphasis

Osler described the typical coronary patient as a "keen and ambitious man the indicator of whose engines is always set at Full speed ahead " Dunbar (20) and Arlow (3) both concluded that a clear cut personality pattern can be detected in many individuals with coronary artery disease characterized among other things by habitual overdrive and compulsive drive to success by means of hard work and self discipline Physiological studies (40) of coronary patients have confirmed this in part In this regard it is also of interest that among Russek s (62) 100 patients with myocardial infarction suffered prior to age 40 most were *either holding down two jobs or working more than 60 hours a week* at one job just prior to their attack Russek emphasized their personality as being aggressive and ambitious with an intense physical and emotional drive a description much like that noted five decades earlier by Osler

Western man has been progressively immersed in a chronically stressful environment increasingly characterized among many attributes by mechanized speed and competitive haste ambitious striving multiple daily work deadlines imparting a constant sense of temporal urgency by the inadequacy of frequently deceptive goals and by increasing

economic debt and frustration leading to extreme and long continued physical and mental effort It seems almost incredible to us that those general forms of socioeconomic stress *unique* to Western societies have been confused with superficial anxiety fear worry or other neurotic or even psychotic states and as a result that emotional stress has been discarded by some as a possible pathogenetic factor in clinical coronary disease on the basis that the latter s incidence is either less or at least not more prevalent in psychotics in primitive races with "tabus and terrifying witchcraft" in suicide prone groups (69) and in the starving Chinese coolie (4 48) and because a temporarily lessened coronary mortality is found in war besieged nations (48 69) Stewart (70) has succinctly differentiated the unique socioeconomic stress of Western man from that of flood famine and pestilence by pointing out that the latter are emotionally accepted since they are outside the individual s control and since in all forms of *group* emotional stress such as war the *individual's* stress is either submerged or lost

The proposal that the occupational and other socioeconomic stress of certain classes of Western man may be a major factor in his relatively high incidence of clinical coronary disease is not without documentation In previous reviews of available epidemiological data (31 33) we have emphasized that if the absence of socioeconomic stress is found in industrialized societies is used as a standard of comparison a better correlation can be obtained with it and the decreased incidence of clinical coronary disease than can be obtained with a low fat dietary intake Brock and Bronte Stewart (7 8) pointed out in their studies in Cape Town that if job responsibility were used as an index as good a correlation could be found between it and the increased incidence of myocardial infarction as was found with the dietary fat intake In England a considerably greater incidence of myocardial infarction has been noted among professional executive and skilled labor groups compared to those of less skilled or unskilled occupations (50 58 63 70 82) Yet among English physicians a sharp rise in myocardial infarction since 1946 has occurred only among the general practitioner group suddenly compelled by the then new system of medical practice to treat a remarkable number of patients in the course of a day s activities (50)

In the study of Gertler and White (34) as well as that of Yater and associates (80) although no difference was found between the dietary habits of their young coronary patients and those of the controls considerable difference was found in the type of civil positions held by the two groups the young coronary patients almost always having occupied a position of responsibility and frequently associated with occupa

tional stress The relatively higher incidence of myocardial infarction in those engaged in managerial executive and professional occupations compared to less skilled and unskilled occupations is further documented by the study of deaths in Philadelphia among white men (37) by the study of Smith and associates (67) by the data of Ryle and Russell (63) and that of Brown and co-workers in England (10) by the study of Pedley (55) and others (57) The marked geographic variation in incidence of mortality from coronary disease in this country may well be due to differences in occupational stress (66) considerably higher mortality rates being found in the densely populated areas and in urban areas as contrasted to rural areas (26 35 66) these differences being similar for both sexes (26)

If chronic exposure to those forms of socioeconomic stress unique to industrialized societies is a major pathogenetic factor in the increasing incidence of clinical coronary disease several possible mechanisms might be operative alone or synergistically First the effects of emotional stress on cardiovascular hemodynamics are profound, and there is much evidence that organic vascular damage may eventuate from such effects (6 9 40 57 78) perhaps in part due to endocrine engendered angiotoxic effects (57 77) the resulting vascular damage predisposing and perhaps in part preceding subsequent lipid deposition of greatest severity in the presence of elevated plasma lipid content (31 33) A second possible mechanism implicates the direct effects of emotional stress upon the plasma lipids

Both qualitative and quantitative aspects of cholesterol metabolism are of probable importance in the pathogenesis of atherosclerosis (33 57) Previous studies (14 33) from our laboratory have indicated that in experimental animals an elevated plasma cholesterol appears to be a passive plasma retention secondary to the intravascular accumulation of an excess increment either of triglyceride or of phospholipid In the normal animal an infusion of triglyceride sufficient to effect a sustained hypertriglyceridemia invariably is associated with a proportionate rise both of plasma phospholipid and of cholesterol (28) apparently due to a passive intravascular sequestration consequent to their preferential solubility in the increment of excess plasma triglyceride (2 11 28) In analogous fashion the rise of plasma cholesterol and of phospholipid in nephrotic rats (Fig 1) and in rats injected with Triton WR 1339 (Fig 2) appears in major part to be a passive secondary accompaniment of the hypertriglyceridemia respectively caused by the deficiency of circulating albumin (59 61) or by the detergent action of Triton (13) It seems likely to us that a similar sequential mechanism pertains in diabetic and in idiopathic hyperlipemia

The above states are of course characterized particularly by a marked increase of plasma triglyceride On the other hand a proportionate rise of plasma cholesterol also occurs in the normal animal when a sustained hyperphospholipemia is induced by the continuous infusion of many although not of all phosphatides (12 29) In biliary obstructive states the hypercholesteremia also appears to be a passive accumulation again secondary to the hyperphospholipemia which is in

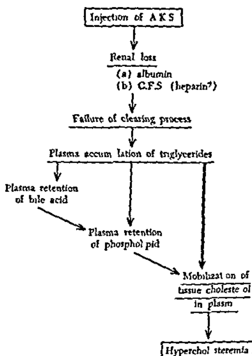


FIG 1 The mechanism of hypercholesteremia in rats made nephrotic by injection of antikidney serum

turn the apparent consequence of the plasma retention of an excess of bile acid a sequence reproducible in the normal animal by an infusion of cholic acid (30) In these instances the plasma triglyceride content is not elevated

The above studies of the interrelationships of the plasma lipids in various hypercholesteremic states imply an important role of the phosphatides in the control of the cholesterol content in normal plasma The major influence of emotional stress on the control of the plasma cholesterol content is shown by recent and current studies in our laboratory Thus the probable important role of hormonal and possibly neurohumoral influences in the control of the plasma cholesterol content is

strongly suggested by the demonstration that various types of emotional stress are uniquely capable of inducing significant and occasionally striking rises of plasma cholesterol From January 8 to June 10 1957 we bled a group of 40 male proprietor accountants biweekly for plasma cholesterol (32 60) Each subject was closely followed at each sampling as regards weight diet exercise and the occurrence of occupational and avocational emotional tension Complete food diaries were kept during two separate 7 day intervals On the basis of their work the accountants were divided into two groups of comparable size and age

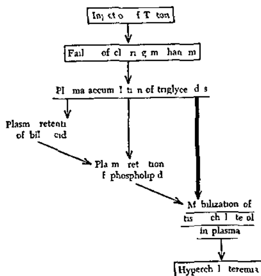


FIG 2 The mechanism of hypercholesteremia in rats injected with Triton WR 1339

(Fig 3) Thus although severe occupational stress was encountered in both groups during the April tax deadline only the corporate accountants (Fig 3 Group B) incurred a comparably severe work stress during the January sampling period The temporal changes of the average plasma cholesterols of the two groups are shown in Fig 4 being found to rise with the occurrence of occupational stress Thus a significant rise of average plasma cholesterol occurred in both groups coincident with the severe occupational stress of the April tax period but only the corporate accountants (Group B) exhibited a similar rise of average cholesterol in January again coincident with this period of severe work stress The fluctuations were not found due to any changes in weight exercise or dietary intake of calories or of fat (32 60)

Significant rises of plasma cholesterol also were observed in many of the subjects at times of unusual personal stress of an avocational nature Nine illustrative examples are shown in Fig 5 in which the occurrence of a significant and sometimes profound rise of cholesterol was closely correlated with the occurrence of severe emotional stress and conversely a significant lowering of cholesterol with the sub

Group	Type	No	Aver Age	Aver Weight Jan 8	Aver Weight Apr 15	Aver Weight June 10
A	TAX	18	38 (28 50)	182 (140 239)	181 (140 239)	182 (140 239)
B	CORPORATE	22	39 (28 56)	175 (147 196)	174 (147 193)	174 (146 193)

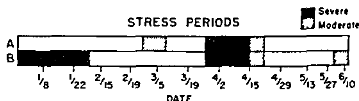


Fig 3 Description of 40 accountants divided into two groups of comparable size and age

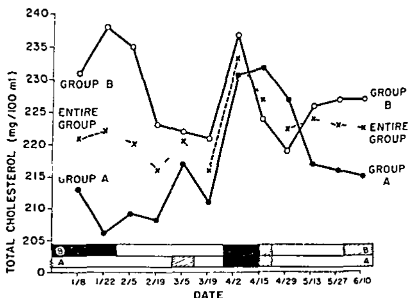


Fig 4 Average serum cholesterol during experimental interval on accountants

sidence of the emotional stress Figure 6 illustrates this particularly well showing the plasma cholesterol changes observed in a 43 year old accountant with xanthelasma and who unknown to us kept a daily record of his exposure to emotional stress of any origin using his own arbitrary unit system Over all plasma cholesterol fluctuations as great as 125 mg per 100 ml were observed to occur in these subjects How

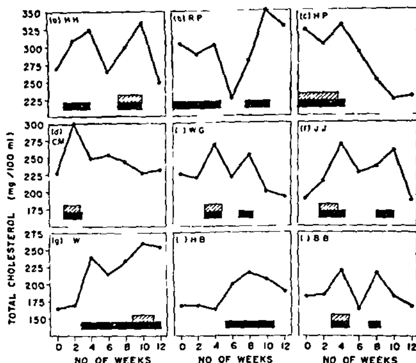


FIG 5 Plasma cholesterol changes in 9 accountants during periods of severe occupational (solid black) and avocational (diagonal lines) stress

ever since these rises occurred in some at times of severe avocational stress as well as during periods of severe occupational tension the cholesterols also were correlated at the time each subject felt his emotional stress from any cause was both minimal and maximal during the experimental period As shown in Fig 7 the differences at such times were not only highly significant but with rare exception each subject's highest plasma cholesterol was observed at the time the subject himself felt his emotional stress to be at its peak and conversely his lowest cholesterol was observed at a time each felt himself to be relatively free of any emotional stress

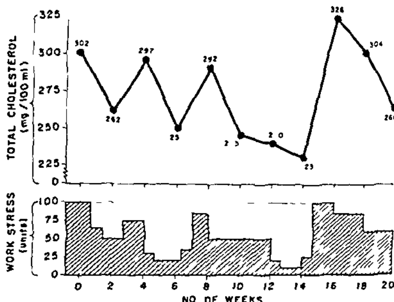


FIG 6 Correlation of serum cholesterol with accountants own diary of work stress

Epidemiologists in general have ignored demonstrations (43 51) of the inconstancy of the plasma cholesterol That the plasma cholesterol is uniquely sensitive to the occurrence of severe mental stress already has been confirmed by several groups of investigators in normal military personnel (27) *in patients with healed myocardial infarction* (36) and

Serum cholesterol at time of		Actual range of each subjects serum cholesterol
Maximum stress		Maximum serum cholesterol
Average	252 mg/100 ml	263 mg/100 ml
Range	(145–391)	(160–391)
S E Mean	± 7 5	± 8 8
Minimum stress		*Minimum serum cholesterol*
Average	210 mg/100 ml	200 mg/100 ml
Range	(138–354)	(127–314)
S E Mean	± 6 8	± 5 7
Difference		Difference
Average	42	63
Range	(2–125)	(31–125)
S E diff means	± 10 1	± 10 5

FIG 7 Average serum cholesterol at time of subjects maximum and minimum period of emotional stress (all types)

in college students during examination week (76) An hypothalamic influence is possibly suggested by the rapidity of the observed rises in certain instances and since exhibition of central nervous system stimulants both experimentally and clinically induces a rise of plasma cholesterol with a lowering occurring during sedative therapy (47 52) Immersion in ice water also is claimed to induce a prompt rise of plasma cholesterol (42) Finally some correlation of plasma cholesterol with blood pressure levels has been observed in normal subjects (46) Epinephrine secretion also must be suspected as a possible factor in the plasma cholesterol response to emotional stress Epinephrine exerts a catabolic (25) and mobilizing (79) effect on lipids (25) in dogs (41) and rabbits (21 22) the repeated administration of epinephrine results in a sustained rise of all plasma lipid fractions However epinephrine particularly affects the triglyceride fraction and it is likely that the rise of plasma cholesterol induced by epinephrine is secondary to the more marked rise of plasma triglyceride

Because of its activation during emotional stress the adrenal cortex is also implicated in the plasma cholesterol response to stress In the rat physiological doses of cortisone fail to affect the plasma cholesterol but as shown in Table I the exhibition of larger doses rapidly increases the plasma content of all lipids particularly that of the triglyceride fraction and also markedly increases the degree of hypertriglyceridemia otherwise induced by olive oil feedings The effect of ACTH (adrenocorticotropic hormone) and adrenocorticoids in ordinary dosage on the plasma cholesterol of humans is not entirely clarified Nevertheless in connection with the significant and sometimes striking rise of plasma cholesterol observed in students during examination week (76) it is of interest that among students exhibiting an anxiety reaction at such times a significant rise of aldosterone and 17 hydroxycorticoid excretion occurs (72)

Although the causal mechanism has not been elucidated and although the role of hormonal secretion in this regard has not been clarified it seems clear that a rise of plasma cholesterol often of profound degree *may* occur in response to unusual emotional stress As a possible corollary it may be proposed (32) that chronic exposure to occupational and other forms of socioeconomic stress unique to Western civilization may be one of the factors in the higher average plasma cholesterol levels found in Western man relative to members of nonindustrialized societies If this proposition were valid it should be possible to demonstrate that among similar age groups with comparable patterns of exercise and diet, the average plasma cholesterol is significantly higher in those chronically exposed to severe occupational stress than in groups free

TABLE I

EFFECT OF CORTISONE ON PLASMA LIPIDS OF RATS

Diet supplement	Cortisone	No of rats	Average plasma concentrations (mg/100 ml)[c]			
			Total lipids	Total cholesterol	Phospho lipids	Triglyceride
Olive oil[b]	—	7	384 (287–617)	67 (55–77)	150 (119–175)	167 (66–365)
Olive oil	+	8	2134 (1020–6100)	139 (96–212)	293 (217–472)	1702 (717–5416)
None	+	5	727 (622–936)	115 (85–145)	233 (192–282)	379 (313–509)

[a] 10 mg subcutaneously daily
[b] 3 ml twice daily
After 3 days treatment

of such stress Unfortunately our current investigations of this problem are as yet too incomplete to present at this time However it is of interest in this regard that in the cooperative study of plasma lipids in normal American subjects (46) it was found that some rise of average cholesterol levels was noted in persons with higher blood pressures and conversely that a low blood pressure level was associated with a low plasma cholesterol The latter correlation was particularly striking in the instance of penitentiary inmates a group of course beset with various emotional problems but nevertheless uniquely "sheltered" from the effects of socioeconomic and other occupational stress and in particular from the pressures of "lack of time" Thus in contrast to the general similarity of the plasma cholesterol levels determined among large groups of different men from various parts of the country the inmates of two different penitentiaries consistently exhibited significantly lower plasma cholesterols not ascribable to dietary differences It is therefore perhaps significant that in a recent study (44) in which normal prisoners were used as controls it was found that the prison inmates exhibited consistently low levels of adrenocortical excretion

A third possible mechanism by which the exposure to emotional stress might affect the coronary arteries is by precipitating thrombotic occlusion Thus although *clinical* coronary heart disease is of course a consequence of atherosclerosis its occurrence is not only the end result of progressive atherosclerotic changes It therefore appeared important to discuss the possible role of emotional stress in *precipitating* clinical disease since this may well be mediated by other than stress induced hemodynamic effects possibly affecting intimal hemorrhage or rupture of or hemorrhage into an atherosclerotic plaque

It must be emphasized that although coronary atherosclerosis is ubiquitous in Western man clinical coronary heart disease and in particular the occurrence of myocardial infarction is frequently engendered by a complication of the atherosclerotic substrate coronary thrombosis Indeed the inference in many epidemiological and other studies that the incidence of myocardial infarction entirely reflects the incidence and the severity of the atherosclerotic substrate would appear to be in part erroneous (31 33) and the occurrence of thrombosis is not linearly related to the severity of the underlying atherosclerotic changes (68)

The importance of the thrombotic factor in the incidence of coronary disease morbidity and mortality is further shown by the fact that the rather rapid decline in mortality from this cause in wartime Scandinavia (56 71) appears to be ascribable primarily to a decreased incidence of all thromboembolic phenomena (19 71) It is also attested to by the success of sustained anticoagulant therapy in preventing myocardial in

farction in patients with known severe coronary atherosclerosis The dissociation between the underlying atherosclerosis and the occurrence of clinical coronary disease is less well indicated by the fact that, even *among certain groups in whom myocardial infarction is almost unknown* some degree of atherosclerotic changes are not so rare (5, 24 38 39) Finally the increase of myocardial infarction in this country and in England in the past 5 decades *is seemingly more ascribable* to an increased incidence of coronary thrombosis (49, 68) than to any significant increase either in the incidence or severity of the atherosclerotic changes (45 49 64) Nor can it seemingly be ascribed to any significant increase either of the plasma cholesterol or of the dietary fat intake (53 54)

It has long been recognized that emotional stress significantly accelerates blood coagulation (16, 65) and there is reason to believe that this may be mediated by an augmented secretion of epinephrine (73 74) and possibly also of adrenal corticoids (15 18) The occurrence of coronary thrombosis and myocardial infarction at times of emotional stress is so well documented (1 17 23 57 75) that it would appear foolhardy simply to ignore its possible causal role in such instances It is therefore of some significance that we have recently observed that an acceleration of blood coagulation occurs at times of severe occupational stress Thus in the study of 40 accountants referred to above (32, 60) blood clotting times were obtained at monthly intervals under carefully controlled circumstances As shown in Fig 8 a significant acceleration of average clotting time occurred in both groups of subjects during the period of severe work stress induced by the April 15 major tax deadline It seems possible therefore that chronic exposure to severe occupational stress unique to those groups of Western man with the highest incidence of clinical coronary disease may increase the hazard of the thrombotic complication by effecting a more or less sustained acceleration of blood coagulation On the basis of current investigations we have reason to believe that this may in part be true but the results of these studies are as yet too incomplete to present at this time

There is an increasing body of evidence (31 33 53 54 81 82) disputing the *solitary* role of dietary lipids in the pathogenesis of coronary heart disease On the basis of clinical and experimental evidence the possible role in the pathogenesis of his increasing clinical coronary disease of the socioeconomic and emotional stress unique to Western man should no longer be neglected Thus if coronary atherosclerosis and thrombotic occlusion are a function of time intimal derangement quantitative and qualitative plasma lipid abnormality hemodynamic

factors and blood coagulation it has been shown that emotional factors are capable of adversely affecting each of these

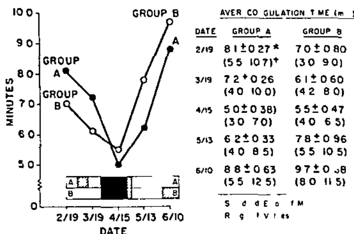


FIG 8 Average blood coagulation times of two groups of accountants

REFERENCES

1 Abendroth H *Munch med Wochschr* 99 178 (1957)
2 Albrink M J Glenn W W L Peters J P and Man E B *J Clin Invest* 34 1467 (1955)
3 Arlow J A *Psychosomat Med* 7 195 (1945)
4 Arnott W M *Lancet* i 1251 (1954)
5 Becker B J P *S African J Med Sci* 11 97 107 (1946)
6 Boshes B *Am J Cardiol* 2 212 (1958)
7 Brock J F and Bronte Stewart B Symposium on Arteriosclerosis of the Minnesota Heart Association p 102 (1955)
8 Bronte Stewart B Keys A and Brock J F *Lancet* ii 1103 (1955)
9 Bronte-Stewart B and Heptinstall R H *J Pathol Bacteriol* 68 407 (1954)
10 Brown R G Davidson L A G McKeown T and Whitfield A G W *Lancet* ii 1073 (1957)
11 Byers S O and Friedman M *J Clin Invest* 35 405 (1956)
12 Byers S O and Friedman M *Proc Soc Exptl Biol Med* 92 459 (1956)
13 Byers S O and Friedman M *Am J Physiol* 190 439 (1957)
14 Byers S O Friedman M and Rosenman R H *Metabolism Clin and Exptl* 1 479 (1952)
15 Caltabiano S and Cagianelli M A *Minerva med* 48 2825 (1957)
16 Cannon W B and Mendenhall W L *Am J Physiol* 34 251 (1914)
17 Chambers W N and Reiser M F *Psychosomat Med* 15 38 (1953)
18 Cosgriff S W *J Am Med Assoc* 147 924 (1951)
19 Dedichen J Strom A Adelsten Jensen R and Closs K *Trans 5th Josiah Macy Jr Conf on Factors Regulating Blood Pressure* p 117 (1951)
20 Dunbar H F Emotions and Bodily Changes" Columbia Univ Press New York 1935

21 Dury A *Circulation Research* 5 47 (1957)
22 Dury A and DiLuzio N R *Am J Physiol* 182 45 (1955)
23 Ecker A, *Ann Internal Med* 40 49 (1954)
24 Elliot G A *Leech* 23 25 (1953)
25 Ellis S *Pharmacol Revs* 8 485 (1956)
26 Enterline P E and Stewart W H *Public Health Repts* 71 849 (1956)
27 Fat and Stress (Col Marshall E Croover quoted) *Time Magazine* p 79 (Jan 28 1957)
28 Friedman M and Byers S O *Proc Soc Exptl Biol Med* 90 496 (1955)
29 Friedman M and Byers S O *Am J Physiol* 186 13 (1956)
30 Friedman M and Byers S O *Am J Physiol* 191 551 (1957)
31 Friedman M and Rosenman R H *Circulation* 16 339 (1957)
32 Friedman M and Rosenman R H *Circulation* 17 852 (1958)
33 Friedman M Rosenman R H and Byers S O *J Gerontol* 10 60 (1955)
34 Gertler M M and White P D Coronary Heart Disease in Young Adults Harvard Univ Press Cambridge Massachusetts 1954
35 Gover M and Pennell M Y *Public Health Repts* 65 819 (1950)
36 Hammarsten J F Cathey C W and Wolf S *J Clin Invest* 36 897 (1957)
37 Hedley O F *Public Health Repts* 54 972 (1939)
38 Holman R L McGill H C Jr Strong J P Griffin O R and Geer J C *Federation Proc* 16 360 (1957)
39 Hyman A S *Ann Internal Med* 22 639 (1945)
40 Jacobson E *Cardiologia* 26 83 (Fasc 2) (1955)
41 Kaplan A and Gant M *Federation Proc* 14 82 (1955)
42 Kuhl W J Jr Beck E M Gershberg H Street E and Ralli E P *Metabolism Clin and Exptl* 4 143 (1955)
43 Kyle L H Hess W C and Walsh W P *J Lab Clin Med* 39 605 (1952)
44 Laidlaw W M Moore D J and Heller C G *Clin Research* 6 55 (1958)
45 Lee K T and Thomas W A *AMA Arch Internal Med* 97 421 (1956)
46 Lewis L A Olmsted F Page I H Lawry E Y Mann G V Stare F J Hanig M Lauffer M A Gordon T and Moore F E *Circulation* 16 227 (1957)
47 Miasnikov A L *Klin Med (USSR)* 34 65 (1956)
48 Mode of Life and the Prevalence of Coronary Heart Disease *in* Symposium on Arteriosclerosis of the Minnesota Heart Association p 28 (1955)
49 Morris J N *Lancet* i 1 (1951)
50 Morris J N Heady J A Raffle P A B Robert C G and Parks J W *Lancet* ii 1053 1111 (1953)
51 Morrison L M Gonzales W T and Hall L *J Lab Clin Med* 34 1473 (1949)
52 Myasnikov A L *Circulation* 17 99 (1958)
53 National Dairy Council How Americans Use Their Dairy Foods National Dairy Council Chicago 1954
54 Page I H Stare F J Corcoran A C Pollack H and Wilkinson C F Jr *Circulation* 16 163 (1957)
55 Pedley F G *Can Med Assoc J* 46 147 (1942)
56 Pihl A *Scand J Clin & Lab Invest* 4 122 (1952)
57 Raab W Hormonal and Neurogenic Cardiovascular Disorders Williams & Wilkins Baltimore Maryland 1953

58 Registrar General Decennial Supplement England and Wales 1951 Occupational Mortality Part I p 13 H M Stationery Office London
59 Rosenman R H Byers S O and Friedman M *J Clin Invest* **36** 1558 (1957)
60 Rosenman R H and Friedman M *Abstr Circulation* **16** 930 (1957)
61 Rosenman R H Friedman M and Byers S O *J Clin Invest* **35** 522 (1956)
62 Russek H I Interview in *Modern Med* June 1 1957
63 Ryle J A and Russell, W T *Brit Heart J* **11** 370 (1949)
64 Saphir O Ohringer L and Silverstone L H *Am J Med Sci* **231** 494 (1956)
65 Schneider R A and Zangari V M *Psychosomat Med* **13** 289 (1951)
66 Sigler L H *Am J Cardiol* **1** 176 (1958)
67 Smith C Sauls H C and Ballew J *Ann Internal Med* **17** 681 (1942)
68 Spain D M Bradess V A and Huss G *Ann Internal Med* **38** 254 (1953)
69 Sprague H B *Circulation* **17** 1 (1958)
70 Stewart I McD G *Lancet* ii 867 (1950)
71 Strom A and Jensen R A *Lancet* i 126 (1951)
72 Venning, E H Dyrenfurth I and Beck J C *J Clin Endocrinol and Metabolism* **17** 1005 (1957)
73 Vosburgh C H and Richards A N *Am J Physiol* **9** 35 (1903)
74 Waldron J M *J Appl Physiol* **3** 554 (1951)
75 Weiss E Dlin B Rollin H R Fischer H K and Bepler C R *A M A Arch Internal Med* **99** 628 (1957)
76 Wertlake P T Wilcox A A Haley M I and Peterson J E *Proc Soc Exptl Biol Med* **97** 163 (1958)
77 Wexler B C and Miller B F *Circulation* **16** 950 (1957)
78 Wilens S C *Am J Pathol* **19** 293 (1943)
79 Wool, I J Goldstein M S Ramey E R and Levine R *Am J Physiol* **178** 427 (1954)
80 Yater W M Traum A H Brown W G Fitzgerald R P Geisler M A and Wilcox B B *Am Heart J* **36** 334 481 683 (1948)
81 Yerushalmy J and Hilleboe H E *N Y State J Med* **57** 2343 (1957)
82 Yudkin J *Lancet* ii 155 (1957)

Discussion

Oliver I am a little hesitant to report the work of somebody else but since Dr Morris work has been considered in some detail today I think that it is important to mention three points which I am quite certain he would discuss if he were here today The first of these is that the social incidence of coronary disease is lost if physical activity is taken into account This Morris will shortly be reporting and I think will interest you The second point is that earlier today there was discussion about the relationship of physical activity and stress in bus drivers and bus conductors in London It was mentioned that bus drivers are more liable to develop myocardial infarction and bus conductors are more liable to develop angina This has been related to their physical activity There are other factors which Morris has subsequently discovered and if he has not already reported this he will be doing so One of these is that the uniform size of the bus drivers and the bus conductors is quite different on entering the service The bus drivers are fatter and

shorter and the bus conductors are leaner. There is somato type difference from the start. The third point is that it is standard practice in London for conductors to be promoted to drivers if they so desire. So that the whole question of physical activity in relationship to stress in these particular investigations should remain *sub judice*. I am quite sure that Morris would like this stated.

ROSENMAN: Interpreting the literature is difficult enough and I would prefer not to comment on unpublished data before examining it more critically.

KATZ: I would like to discuss several things that Dr. Rosenman presented on behalf of himself and Dr. Friedman. First, I am delighted that in this presentation emphasis is placed on the fact that stress as defined in San Francisco (as contrasted with that in Quebec) is one of the possible factors, not the only factor responsible for coronary episodes. It also is pleasing that diet is not denied a possible role. The second thing I would like to point out is that there are three aspects to stress as I interpret the presentation. One is the rather exciting work on the income tax accountants on blood cholesterol and blood clotting during a period of high peak load. I think these facts require no comment. Two, there is the implication that emotional stress universally acts on certain males and has a cumulative effect. As a postulate it is interesting, but it should be proven. Third, there is a vague and vascillating picture concerning the susceptible personality types. This too is challenging, but not proven, as I will point out tomorrow. But what I want to put publicly into the record is that the time is ripe for Dr. Friedman and Dr. Rosenman to deal prospectively with their interesting idea, to wit: (*a*) define beforehand the special personality type, perhaps in a number of groups; (*b*) define in advance the several types of stress loads. Then they should carry out a 5- or 10-year follow-up on these people to see whether or not their ideas are confirmed.

ROSENMAN: Thank you for your comments, Dr. Katz. We are even now engaged in studies and in designing further investigations along the very lines in part that you have suggested. However, if atherogenesis is variously a function of time, hemodynamic factors, intimal damage and disturbed lipid metabolism, and if myocardial infarction is in many instances a function of thrombotic occlusion superimposed on an atherosclerotic substrate, then it already has been demonstrated by various data presented at this conference as well as by many other studies that each of these factors is adversely influenced by exogenously administered adrenal hormones. On the other hand, it is well shown that emotional stress stimulates adrenal secretion, or in other words, that cerebral cortical perturbation stimulates a self-engendered administration of endogenously derived adrenal hormones. In the presence of emotional tension there may be the associated factors of diminished exercise and augmented dietary intake as well as altered blood flow. We would suggest the following diagram to express these relationships and hypothesize that occupational stress in susceptible individuals, as increasingly found in middle and upper class males of industrialized societies, more or less continuously stimulates such cortical perturbation. (Fig. A)

FREEDBERG: I can confirm Dr. Oliver's remarks with regard to Dr. Morris' work. And to add to the statistical evaluation, perhaps you can tell us what Dr. Yudkin said about the relationship between the sale of television sets and the incidence of deaths from acute myocardial infarction in the same paper you referred to.

ROSENMAN: I believe Dr. Yudkin found in the study referred to by Dr. Freedberg, that the increase of mortality from coronary heart disease in England has parallelled the increase of television sets and automobiles far more closely than it has any change in the diet.

FURMAN One of the criticisms of studies which relate a low fat intake in certain population groups during World War II to a reduced mortality from coronary artery disease is that the reduction in death rate occurred prior to fat restriction (taking England as an example) and later on the death rate from coronary artery disease began to rise and approach the rate characteristic of the country prior to the war even though meanwhile the fat restriction became more stringent I was wondering how this rise in death rate due to coronary artery disease during the course of the war fits in with your concept of the role of stress in this regard

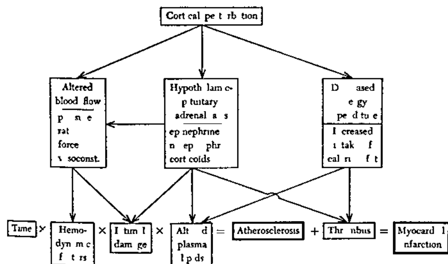


FIG 4 Diagram illustrating possible pathways by which cortical perturbation may participate in mechanism of myocardial infarction

ROSENMAN I am not familiar with data from wartime Britain I believe Pihl (*Scand J Clin & Lab Invest* 4 12_ 1952) found the fall in wartime Norway's mortality from arteriosclerosis to occur prior to the decreased fat consumption Scrutiny of Malmros data (*Acta Med Scand Suppl* 246 137 1950) indicates that the decreased mortality from arteriosclerosis in 1940 in Norway was associated with decreased fat consumption with a rise of both in 1944 on However a falling mortality in Norway from arteriosclerosis can be noted in 1935 to 1939 despite an increased fat intake during these years In Denmark the mortality from arteriosclerosis was unchanged from 1939 to 1945 and decreased from 1946 to 1948 although the total fat intake decreased progressively from 1939 to 1948 In Finland the mortality from arteriosclerosis increased from 1938 to 1940 during a period of decreased fat intake and although the latter continued to decrease from 1940 to 1947 the mortality from arteriosclerosis decreased only from 1940 to 1943 and then progressively increased In Sweden a progressive increase in mortality from arteriosclerosis occurred in 1935 to 1940 although there was no associated increase in fat consumption during these years

Thus I believe it is an error to lift the wartime experience in Norway out of the general context as has so commonly been done Moreover as Dedichen Strom

and Jensen (*Trans 5th Josiah Macy Jr Conf on Factors Regulating Blood Pressure* 1951) have pointed out the fall of mortality from arteriosclerosis in Norway in 1940 and its later rise in 1946 so closely paralleled the fall and lat r rise of fat consumption that it would seem extremely hazardous to ascribe the altered mortality to any sudden resolution and later increased incidence or severity of atherosclerosis effected by the varying fat intake

During wartime there is an altered pattern of emotional stress the individual's emotional stress being transferred to and submerged in the national group stress I do not know whether this played a causal role in the changed mortality rates in wartime Norway or whether the decreased fat intake at this time decreased the hazard of the thrombotic complication of the arteriosclerotic vessel However the data of Dedichen Strom and Jensen appear to indicate clearly that the decreased mortality from arteriosclerosis in wartime Norway was associated with and ascribable to a closely parallel decrease of all thrombotic accidents

CHAPTER 22

Influences of Hormones on Circulating Lipids[1]

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Hormonal influences on carbohydrate and protein metabolism have been extensively studied In contrast information concerning hormonal influences on lipid metabolism is limited although evidence is accumulating that such mechanisms exist and probably are important (3)

The concept that atherosclerosis is a metabolic disorder (1) involving chiefly lipids and lipoproteins has stimulated extensive investigation of the endocrine influences on circulating lipids and on lipid metabolism Even in countries with low dietary fat consumption there is still an individual susceptibility to atherosclerosis which cannot be explained by environmental factors only There is evidence that hereditary influences determine the individual susceptibility to atherogenesis presumably through metabolic mechanisms and through genetically controlled enzymatic reactions (2) The endocrine system may play an important regulatory or mediating role in these mechanisms

THE THYROID

The role of the thyroid in the control of serum lipids is better established than that of the other endocrine glands Total thyroidectomy in man and in experimental animals results in a decided elevation of serum lipids According to our own studies the elevation of the individual lipid fractions varies with the species (Table I) In rabbits there is a moderate increase in serum cholesterol affecting total and esterified cholesterol approximately equally (+ 54% and + 47% respectively) in serum phospholipids (+ 30%) and in serum triglycerides (+ 178%) the more pronounced rise in triglycerides is mainly responsible for the increase of serum total lipids (+ 103%) The failure of thyroidectomy to produce marked elevation of serum cholesterol in rabbits and to enhance atherogenesis was previously reported by Turner and Kayat

In contrast dogs after thyroidectomy exhibit a greater elevation of total and esterified serum cholesterol (+ 169 and + 112%) and of serum phospholipid (+ 53%) than of serum triglycerides (+ 10%)

[1] This research was supported in part by grants from the Division of Research Grants United States Public Health Service

TABLE I

EFFECT OF THYROIDECTOMY ON PLASMA LIPIDS IN RABBITS AND DOGS[a] (2)

Animals (no.)	Control				Time after Thyroidectomy (mo.)	Peak values after thyroidectomy				Increase in % of control value			
	Cholesterol total/esterified	Phospholipid	Triglycerides	Total lipids		Cholesterol total/esterified	Phospholipid	Triglycerides	Total lipids	Cholesterol total/esterified	Phospholipid	Triglycerides	Total lipids
Rabbits 20	41/32	87	112	240	2	63/47	113	311	487	54/47	30	178	103
Dogs 9	144/112	290	274	708	2.5	388/238	443	302	1133	169/112	53	10	60

[a] All values are expressed in mg./100 ml.

the increase in serum total lipids (+ 60%) in the dog is mainly caused by the higher levels of serum cholesterol and phospholipid

THE GONADS

Evidence is accumulating that the gonads exert an important influence on the level of circulating lipids and lipoproteins. The comparatively rare occurrence of coronary artery disease in women during the reproductive phase is well known. Bilaterally oophorectomized women show a higher incidence of coronary artery disease than normal women of corresponding ages.

Age and sex affect serum lipid levels in man. Dietary factors, particularly dietary fat, appear to be related to serum lipid concentrations. The role of other factors such as ethnic origin, occupation, stress, physical activity, smoking and alcohol is questionable.

Adlersberg *et al.* (4) examined approximately 1200 healthy males and females between the ages of 2 and 77 years of low middle income in New York. The almost completely white population was otherwise moderately heterogeneous with a distinct predominance of families of Italian and Irish origin. The total serum cholesterol level of the males remained constant from age 2 through 19. From age 20 through 33 there was a significant increase of total cholesterol level averaging 3.6 mg % per year. Thereafter until age 60 there was no further change. The total serum cholesterol level of the females did not change significantly from age 2 through 32 although there appeared to be a slight decrease from age 2 through 20. From age 33 through 58 a significant rate of increase of 3.2 mg % per year occurred (Table II).

The changes in serum phospholipid levels with age were similar to the changes in serum cholesterol levels in the two sexes.

Sex differences in the distribution of serum lipoproteins have been reported by various laboratories using the Cohn protein microfractionation technique, the ultracentrifuge technique and zone electrophoresis with subsequent elution. Normal young women have a relatively greater cholesterol concentration in the serum alpha lipoprotein fraction and correspondingly smaller amounts of cholesterol in the beta lipoprotein fraction than normal men of corresponding ages. This difference is not apparent after the menopause.

Considerable information is available as to the effects of gonadal hormone in man. Eilert reported in 1949 on the effect of estrogens in pre and postmenopausal women. A decided reduction in plasma cholesterol and the cholesterol phospholipid ratio was observed (7). Extensive studies on the effects of estrogens and androgens in patients who survived myocardial infarction were later reported by several investi

gators (9 10 11) Synthetic or naturally occurring estrogens corrected the abnormal lipid and lipoprotein pattern of survivors of myocardial infarction They lowered plasma cholesterol and raised the cholesterol concentration of the alpha lipoproteins and correspondingly diminished the cholesterol concentration of the beta lipoproteins

TABLE II
CHANGES IN SERUM CHOLESTEROL LEVEL WITH AGE IN MALES AND FEMALES (4)

Age interval (yr)	No	b[a]	P[b]
		Males	
2–19	154	−0 278	>0 20
20–33	74	3 622	0 01>P>0 001
34–50	213	−0 445	>0 20
51–60	96	0 821	>0 20
		Females	
2–13	100	−1 478	>0 20
14–20	58	−2 065	>0 20
21–32	104	−1 077	>0 20
33–58	263	3 181	<0 001

[a] The average annual change of total serum cholesterol in mg/100 cc is represented by the coefficient b in the regression equation $Y = a + bX$ where X = age in years and Y = serum cholesterol level

[b] Probability that the true value of the average annual change may be zero

The use of large doses of estrogens such as 1 0 mg estinyl or 10 mg Premarin daily produced severe systemic changes gynecomastia complete impotence restlessness depression and not infrequently nausea and gastric distress Robinson *et al* (10) treated a group of men with myocardial infarction for periods of 6 to 48 months with estrogens [average 10 mg of mixed conjugated estrogens (Premarin)] A decrease in serum total cholesterol with increase in phospholipid and in alpha lipoprotein cholesterol was observed Sexual potency gradually decreased and eventually became absent Gynecomastia proved to be so distressing that in the younger age group surgical removal of the glandular tissue of the breast was performed prior to institution of estrogen therapy

It is of interest that much smaller dosages of estrogens than those mentioned can rectify abnormal serum lipids and lipoprotein patterns and restore them to normal This form of hormonal therapy is associated with less severe adverse effects Oliver and Boyd (8) treated 10 men with daily doses of 0 2 to 0 6 mg of ethinyl estradiol over a period of more than 11 weeks An average depression of total plasma cholesterol of 25% was achieved ($P < 0 01$) the cholesterol phospholipid ratio

showed an average fall of 29% ($P < 0.01$) Even with these smaller doses gynecomastia nausea, dizziness fatigue and depression were common complaints and loss of libido occurred in some subjects

In our laboratory the effects of long term treatment with low doses of ethinyl estradiol (estinyl) on the serum lipids and lipoproteins were studied in patients with idiopathic hyperlipemia and idiopathic hyper cholesteremia (6) Only patients who had been receiving therapy for a minimum of 2 months were included in this report The group in cluded 4 men and 2 women with idiopathic hyperlipemia (average age 48 years) and 2 men and 3 women with idiopathic hypercholesteremia (average age 47 years) Four patients had skin xanthoma, and 2 had xanthoma tendinosum 5 had coronary artery disease and 1 had recur rent acute pancreatitis The average duration of therapy was 8 months (range 2–14 months) Doses ranged from 0 1 to 0.2 mg daily A low fat diet was instituted prior to and during the study The body weight remained stable

In both groups lipid levels were lower under estrogen therapy Maximum effects were noted after approximately 6 weeks (Table III) In the hyperlipemic group levels of serum total and esterified choles terol decreased by 50% and total lipids by 51% The serum phospho-lipids showed a fall averaging 30% The beta lipoprotein fraction rose slightly from 35.5% to 40 4% and the O (origin) fraction decreased from 50 6 to 36 4% There was however a marked increase in alpha lipoprotein from 11 9 to 23.2% of the total stainable lipid

In the hypercholesteremic group levels of serum total and esterified cholesterol decreased, by 35 and 39% respectively and average serum phospholipids by 12% Total lipids fell 21% Beta lipoprotein de creased from 68 9 to 59.2% the O fraction decreased from 16 9 to 9 0 and alpha lipoprotein increased from 14.3 to 31 8% of the total stainable lipid

Levels of lipids and lipoproteins on placebo therapy were unchanged from control levels Moderate gynecomastia and diminished libido were observed uniformly in men In 2 postmenopausal women, mild uterine bleeding occurred In 2 patients the cardiac status improved in 3 it was unchanged In 1 patient with idiopathic hyperlipemia and ex tensive xanthoma tuberosum there was gradual disappearance of the lesions during 14 months of estrogen therapy (see Fig 1)

The Adrenals

The role of the adrenals in regulating circulating lipids and athero-genesis has been discussed in a preceding article (Adrenocortical Hor mones and Atherosclerosis)

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[a] The average annual change of total serum cholesterol in mg/100 cc is represented by the coefficient b in the regression equation $Y = a + bX$ where X = age in years and Y = serum cholesterol level

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The Pancreas

Knowledge concerning the role of the pancreas in controlling the concentration of circulating lipids is based on the study of pancreatitis and diabetes mellitus in both the experimental animal and in man

An early observation concerning transient hyperlipemia and hypercholesteremia in experimental pancreatitis in the dog was reported by Binet and Brock in 1929 (5) In our laboratory studies on the relationship between pancreatitis and serum lipids were performed in rabbits and dogs treated with ethionine a powerful metabolic competitor of methionine This substance produces severe changes of hemorrhagic pancreatitis Pathological changes in many other internal organs are concomitantly produced such as fatty metamorphosis of the liver renal damage and destruction of chief cells of the stomach Progressive diminution of circulating proteins lipids lipoproteins and glycoproteins was seen (13)

Because of the severe pathological changes in many vital organs other than the pancreas and the resulting metabolic disorder it was thought inadvisable to use ethionine produced pancreatitis for study of the relationship between the pancreas and circulating lipids In a search for a more specific "isolated" form of experimental pancreatitis the instillation of a potent staphylococcus toxin into the ligated pancreatic duct (12) proved to be satisfactory The study included 35 rabbits and 11 dogs The full procedure resulted in acute fulminating pancreatitis and produced concomitant lactescence of serum lasting 1 to 4 days (Table IV) The maximum elevation of serum lipids observed on the second day comprised an increase of approximately 200% in total cholesterol and in phospholipid and 400% in total lipids mainly caused by elevation of triglycerides

The serum cholesterol and phospholipid returned sooner toward normal levels than did the triglycerides and total lipids The maximum elevation of the blood sugar and of serum amylase again was noticed on the second postoperative day The control group in which ligation of the pancreatic duct was combined with intraductal instillation of saline solution showed slight elevation of serum lipids no change in blood sugar but significant elevation of serum amylase Similar eleva

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150 and 100 μg and 1 week intermissions results in decided though not maximum, lowering of serum lipids Note reduction of the O-fraction of serum lipoproteins under adequate estinyl therapy and marked increase during low dosage periods (50 μg) and on placebo Note marked reduction of xanthomatous lesions on elbows and knees after 8 and 14 months of therapy The smaller lesions disappeared completely while the larger ones showed marked reduction in size

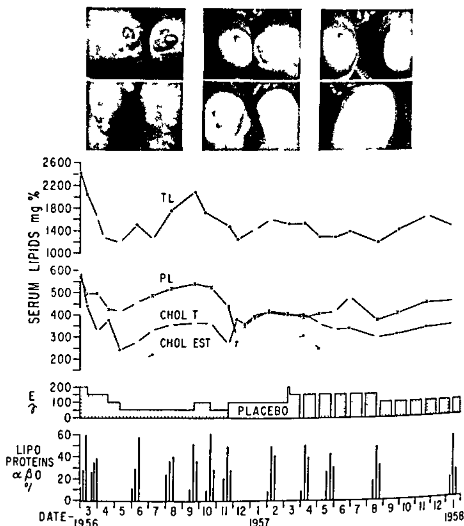


FIG 1 C N 42 year old white man with idiopathic hyperlipemia and xanthoma tuberosum Effect of prolonged estinyl therapy of almost 2 years duration on serum lipids and lipoproteins and on skin xanthomata Throughout observation period the diet remained unchanged

KEY T L — Total serum lipids P L — Serum phospholipids Chol T — Total serum cholesterol Chol Est — Cholesterol esters Serum lipoproteins (at bottom) are depicted as 3 columns representing alpha beta and O (origin) lipoprotein

Xanthomata of the elbows are depicted in the upper row those of the knees in the lower row of photographs Nos 1 and 4 are prior to estinyl therapy Nos 2 and 5 after 8 months of treatment and Nos 3 and 6 after 14 months of treatment

Note marked reduction of all serum lipid fractions following daily intake of 200 and 150 µg of estinyl and definite increase of the levels following both reduction to 50 µg and replacement by placebo The subsequent period of intermittent estinyl administration consisting of 3 week periods of treatment with daily doses of

TABLE IV

EFFECT OF EXPERIMENTAL PANCREATITIS PRODUCED BY PANCREATIC DUCT LIGATION AND INTRADUCTAL STAPHYLOCOCCUS TOXIN INJECTION (Staph T' IN TABLE) ON SERUM LIPIDS OF THE RABBIT IN THE CONTROL ANIMALS ("Saline" IN TABLE) LIGATION OF THE DUCT AND INTRADUCTAL INJECTION OF SALINE SOLUTION WAS USED (2)

Serum components (mg/100 ml)	Animals (no)	Subtance injected into pancreatic duct	Days after ligation of pancreatic duct + intraductal injection										
			0	1	2	3	4	6	8	10	12	14	16
Cholesterol total/	19	Staph T	50/40	54/42	141/69	72/45	53/35	66/47	—	—	—	45/38	—
esterified	4	Saline	50/35	38/24	70/57	42/36	—	50/34	—	41/32	—	—	43/31
Phospholipid	19	Staph T	122	180	340	232	131	162	—	—	—	128	—
	4	Saline	115	155	144	110	—	133	—	133	—	154	—
Total lipids	19	Staph T	263	630	1149	1075	532	430	485	—	—	302	402
	4	Saline	260	273	335	260	—	303	303	348	—	295	295
Blood sugar	19	Staph T	130	125	247	177	—	150	—	—	—	—	202
	4	Saline	—	—	83	107	—	91	89	203	105	—	149
Amylase	19	Staph T	204	184	462	294	267	196	225	—	—	206	169
	4	Saline	202	—	397	383	—	249	—	232	182	—	209

TABLE III

Effect of Prolonged Estinyl Administration (2 to 14 Months Average 8 Months) in Daily Doses of 0.1 to 0.2 mg on Serum Lipids and Lipoproteins (by Paper Electrophoresis) (2)[a]

Condition	No	Average age	Before estinyl: Cholesterol total/esterified	Before estinyl: Phospholipid	Before estinyl: Total lipids	After estinyl: Cholesterol total/esterified	After estinyl: Phospholipid	After estinyl: Total lipids
A Lipids								
Idiopathic hyperlipemia	6	48	584/393	635	2909	290/198	441	1421
Idiopathic hypercholesteremia	5	47	467/358	428	1321	304/217	378	1040
B Lipoproteins								
			Alpha	*Beta*	*O*	*Alpha*	*Beta*	*O*
Idiopathic hyperlipemia	6	18	11.9	35.5	50.6	23.2	40.4	36.4
Idiopathic hypercholesteremia	5	47	14.3	68.9	16.9	31.8	59.2	9.0

[a] Serum lipids are expressed in mg/100 ml serum lipoproteins in percentages of stainable lipid

TABLE V

Effect of Experimental Pancreatitis on Serum Lipids of the Dog[a]

Serum components (mg/100 ml)	Animals (no)	Substance injected into pancreatic duct	Days after ligation of pancreatic duct + intraductal injection							
			0	1	2	3	4	6	9	14
Cholesterol total/esterified	9	Staph T	163/126	216/154	204/139	255/180	295/214	143/112	164/119	125/117
	2	Saline	171/138	149/107	172/130	136/118	—	137/107	154/117	147/116
Phospholipid	9	Staph T	354	369	465	488	469	313	362	339
	2	Saline	340	410	410	410	—	375	346	414
Total lipids	9	Staph T	680	885	1126	970	1078	729	770	661
	2	Saline	765	813	968	770	—	810	765	860
Blood sugar	9	Staph T	106	106	69	88	84	89	89	102
	2	Saline	100	67	83	69	—	90	72	72
Amylase	9	Staph T	350	499	543	935	960	643	638	774
	2	Saline	191	176	134	300	—	524	496	376

[a] For details see Table IV

tion of serum amylase was seen in rabbits in which under anesthesia a simple laparotomy without any additional manipulation was performed as well as in a group of 9 animals in which the pancreatic duct was ligated without any intraductal instillation In none of these control groups were significant changes of serum lipids seen In animals in which the toxin was instilled intraductally 2 weeks after pancreatic duct ligation severe toxic manifestations were observed within the first 24 hours after which time the moribund animals were sacrificed It appears then that acute pancreatitis produced in otherwise normal rabbits results in characteristic transient elevation of serum lipids affecting mainly triglycerides

In dogs experimental pancreatitis produced similar effects It caused elevation of serum lipids for 1 to 4 days but to a lesser degree (Table V)

The mechanism by which pancreatitis leads to the alteration of serum lipids is obscure although several possibilities have been considered Diabetes often is present in experimental and human pancreatitis but it is too mild to explain the elevation of serum lipids Deficiency of a specific pancreatic hormone regulating lipid metabolism (lipocaic) has been previously considered However the existence of this hormone is questionable at present Certain observations point to a possible role of the alpha cells of the pancreas and their hormone glucagon Destruction of alpha cells by cobaltous chloride results in an elevation of serum lipids after 24 hours with gradual return to control levels after 7 days as in experimental pancreatitis The problem whether or not glucagon is involved in the regulation of lipid metabolism requires extensive additional studies It is of interest that addition of glucagon resulted in a decrease of fatty acid synthesis in liver slices incubated with C^{14} acetate One must always remember that hormones affecting carbohydrate and/or protein metabolism such as insulin may indirectly affect lipid metabolism

To summarize, it may be stated that many of the hormones exert marked effects on circulating lipids While some of these influences are well established some others require extensive additional studies

References

1 Adlersberg D *Am J Med* 11 600 (1951)
2 Adlersberg D *Am J Med* 23 769 (1957)
3 Adlersberg D Schaefer L E and Drachman S R *J Clin Endocrinol* 11 67 (1951)
4 Adlersberg D Schaefer L E and Steinberg A *J Am Med Assoc* 162 619 (1956)
5 Binet L and Brocq P *Paris méd* 1 489 (1929)
6 Bossak E T Wang C I and Adlersberg D *Circulation* 16 503 (1957)

temperature While I have not studied the serum cholesterol of such animals it is possible that the effects on the bilirubin and cholesterol metabolism are related to the effect of fever on liver blood flow and hepatocellular function

ADLERSBERG It is quite possible We lost many animals before we were in position to establish an adequate dose for our experiments We started the dilution of a staphylococcus toxin of 1 20 and we lost all the animals in this series Then we diluted again 100% and a dilution of 1 40 of the same toxin also killed the animals A concentration of 1 80 was then used and it was possible to produce severe pancreatitis which the animals survived

BOYD This point which you raised Dr Adlersberg on the possibility of some of the effects of pancreatitis being mediated through glucagon and the alpha cells When Campenhout and Cornelius described the effects of administering cobalt chloride to guinea pigs and showing histological damage to the alpha cells this was taken up by Caren and Carbo who showed that the administration of cobalt chloride to rabbits produces a hypercholesterolemia in 2–3 days These authors concluded that the pancreas secretes a hormone which controls the plasma cholesterol level in the rabbit Dr MacLean and I have looked into this effect and we have administered about 40 mg/kg cobalt chloride to rabbits and examined the pancreas of these animals at various times from 1 hour to several days after the administration of this substance In the rabbit this dose of cobalt chloride does not result in pancreatic alpha cell destruction but produces a very marked hyperglycemia which persists for about 8 hours and a hypercholesterolemia Now if the cobalt acted on the alpha cells and the alpha cells produce glucagon it might be possible to imitate this by giving glucagon to these animals If glucagon is administered a very sharp hyperglycemia ensues quite different from the cobalt hyperglycemia and there is no effect on the blood cholesterol level It could be that the cobalt causes a release of adrenalin producing the hyperglycemia which is superimposable on the cobalt hyperglycemia but the administration of adrenalin to the rabbit does not produce hypercholesterolemia The liver was the only organ which showed consistent histological changes in these cobalt treated animals

ADLERSBERG Thank you very much Dr Boyd I have no personal experience with cobalt chloride I based the speculations that I presented on facts reported in the literature

7 Eilert M L *Am Heart J* 38 472 (1949) *Metabolism Clin and Exptl* 2 137 (1953)
8 Oliver M F and Boyd G S *Am Heart J* 47 348 (1954)
9 *Oliver M F and Boyd G S Lancet* ii 1273 (1956)
10 Robinson R W Higano N Cohen W D Sniffen R C and Sherer J W Jr *Circulation* 14 365 (1956)
11 Russ E M Eder H A and Barr D P *Am J Med* 11 468 (1951)
12 Thal A and Molestine J E *A M A Arch Pathol* 60 212 (1955)
13 Wang C Adlersberg D and Strauss L *A M A Arch Pathol* 65 279 and 286 (1958)

Discussion

Drill Dr Adlersberg what were the estrogenic side effects if any when the low doses of ethinyl estradiol were given?

Adlersberg We have seen the same side effects that have been reported before Most of the men showed breast changes but *none of them* were very seriously perturbed by that Of course diminished libido was a frequent complaint of the patients and of their wives

Hellman Will Dr Adlersberg please comment on the mechanism of the rise in cholesterol observed in these cases of pancreatitis

Adlersberg I am very thankful for this question Dr Hellman because I planned to discuss this point but I did not have time We don t know for sure but one could speculate on some possibilities We believe for instance that the diabetes observed in these animals and in *some patients with pancreatitis does not* explain the serum lipid changes Especially in animals whose data I showed here the elevation of the blood sugar levels was too mild to explain the serum lipid changes Of course one is always inclined to think of lipocaic as a possible factor However since lipocaic is a substance the existence of which is doubtful such an explanation is not valid There is perhaps a possibility that some damage to the alpha cells may be important There are reports that cobalt chloride when injected into guinea pigs produces changes in the alpha cells of the pancreas and transient serum lipid changes *somewhat similar to those observed in pancreatitis* One could speculate that the effect of pancreatitis on the alpha cells and perhaps an increased production or mobilization of glucagon is perhaps responsible for these changes but we have no definite explanation

Hellman *I was particularly interested if there is any evidence of* biliary obstruction or altered liver function in either spontaneous or experimentally produced pancreatitis

Adlersberg All animals were carefully studied at necropsy We found no hepatic damage provided that small doses of staphylococcus toxin were used If you use very large doses then you get terrific toxic effects including shock and drop of blood pressure These animals often did not survive the operation for longer than several hours As a matter of fact you may get similar results I didn t go into that if you ligate the duct first and after the pancreas gets atrophic inject staphylococcus toxin several weeks later intraductally A fulminating form of toxemia and shock kills these animals within a few hours A marked drop in serum lipids may be seen under these circumstances

Freedberg I just wanted to comment on the point that Dr Adlersberg just discussed namely that there is in experimental pancreatitis shock Following staphylococcus toxin *Shigella* endotoxin or other toxins one may see peripheral vascular collapse With small doses one may also see marked elevation of body

CHAPTER 23

Effect of Desiccated Thyroid Substance and Thyroid Congeners upon Serum Lipoproteins and Serum Cholesterol Levels

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I should like to present in rapid succession some of the extensive experience accumulated in the past 4 years at the Donner Laboratory on the effects of thyroid active substances on human serum lipoproteins and on other physiologic parameters

The studies with desiccated thyroid *l* thyroxine and *l* triiodothyronine to be presented differ in three main aspects from most other similar investigations First we have used rather high dosage levels for prolonged periods of time Second we have employed clinically euthyroid patients Third we have studied a population of chronic schizophrenic institutionalized patients having no other associated disease and who have not been treated with any of the modern tranquilizing agents electroshock or other forms of therapy for months preceding our studies The study of such patients made it possible to observe patients in a hospital environment under carefully controlled conditions for prolonged periods of time

To avoid confusion from the number of slides showing the most pertinent results of our studies I would like to begin by stating the main conclusions which emerge from these studies These are

1 The three thyroid active substances studied have a rather specific hypolipoproteinemic effect on the S_f^0 0–12 and S_f^0 12–20 classes of serum lipoproteins

2 Prolonged administration of effective hypolipoproteinemic doses of desiccated thyroid *l* thyroxine and *l* triiodothyronine produces only few and minor side effects

3 The hypolipoproteinemic and other physiologic effects of the above three thyroid active substances are compared for the purpose of showing specific differences and similarities in the effects of these hormones

The first study (10) deals with 19 male schizophrenic patients ages 20–40 who were given 3½ and then 7 grains of desiccated thyroid for 1 week each followed by a 9 week course of 10 grains Only 11 of the 19 patients tolerated this rather arduous course Of the 8 who did not complete the entire course 4 sustained excessive weight loss (defined as in excess of 10 pounds within 2 weeks) 2 showed increases in pulse rate above 100 beats/minute and 2 patients became severely nervous

Figure 1 shows a dramatic lowering of the S_f^0 0–12 serum lipoprotein class with a sharp rebound a somewhat smaller decrease in serum cholesterol levels and a definite decrease in the S_f^0 12–20 levels with smaller reductions in S_f^0 20–100 and S_f^0 100–400 serum lipoprotein concentrations I should like to point out that only at these very high dosage levels are statistically moderately significant ($p < 0.05$) reductions in S_f^0 20–400 serum lipoprotein concentrations observed Changes in S_f^0 0–12, S_f^0 12–20 and serum cholesterol levels are of course statistically

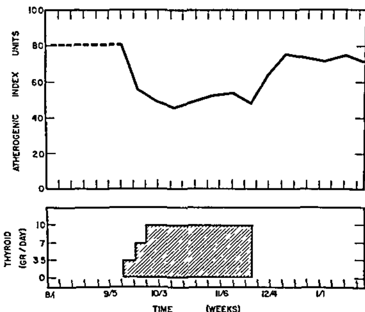


FIG 2 The effect of 10 grains of desiccated thyroid on atherogenic index

very highly significant Average weight loss for the entire course amounted to about 12 pounds Figure 2 shows the very marked decrease in atherogenic index observed in this study

The question naturally arose regarding minimal effective hypolipoproteinemic doses of desiccated thyroid for prolonged use To answer this question the following study was done by Strisower *et al* (11) A group of 60 schizophrenic patients composed of 30 male and 30 female persons both with a mean age of 40 years was given 3 grains of desiccated thyroid for 30 weeks followed by 4 grains for 39 weeks followed by 5 grains for 36 weeks followed by a 27 week follow up period without any thyroid substance This extensive study extended thus over a period of 132 weeks Thirty nine patients completed the entire course and of

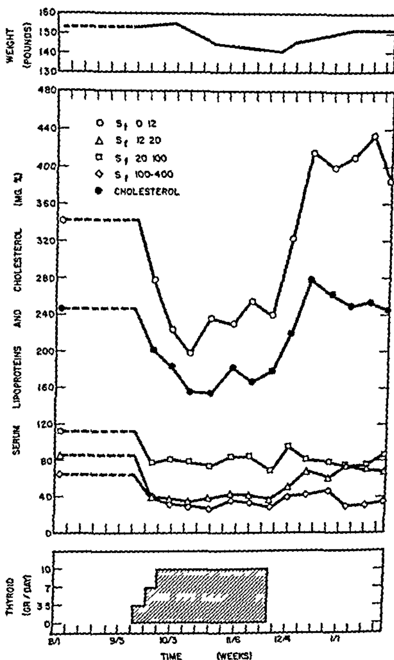


Fig 1 The hypolipoproteinemic effect of 10 grains of desiccated thyroid

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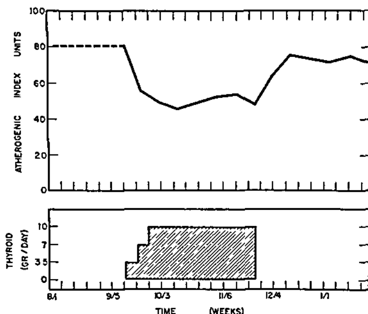


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the 21 patients who did not 6 were discharged 6 were transferred for active psychiatric treatment 1 required a minor surgical procedure, 2 refused venipuncture and only at most 6 were considered to show possible evidence of hyperthyroidism Two of the 6 became increasingly nervous, and 4 lost between 20 and 30 pounds of weight Figures 3 and

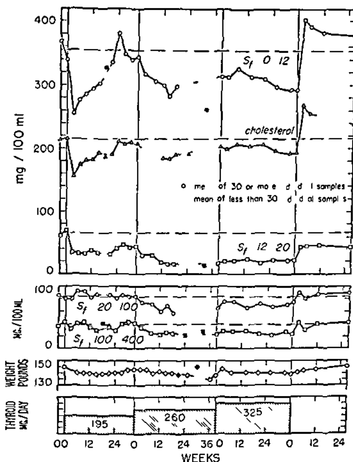


Fig 3 The hypolipoproteinemic effect of the long term administration of 3–5 grains of desiccated thyroid

4 show the main results of this study Figure 3 shows a rapid fall in S_f° 0–12 serum lipoprotein concentrations maximal at about 3 weeks but then escaping" to pretreatment levels during the following 10 to 15 weeks We believe this escape phenomenon is due to a very neat physiologic mechanism applying in this case to a hypolipoproteinemic effect but first shown by Greer (6) based on observations involving I^{131} uptake studies Briefly the maximal hypolipoproteinemic effect ob

served at 3 weeks followed by the gradual escape is consistent with the concept that endogenous thyroidal hormone production about equivalent to 3 grams of desiccated exogenously administered thyroid is gradually completely inhibited by this exogenous thyroid administration This explains the initial sharp drop in S_f^0 0–12 serum lipoprotein concentrations as being due to approximately 6 grams of effective" desiccated thyroid (3 of endogenous and 3 of exogenous origin) the escape and rebound phenomena (following cessation of exogenous thyroid administration

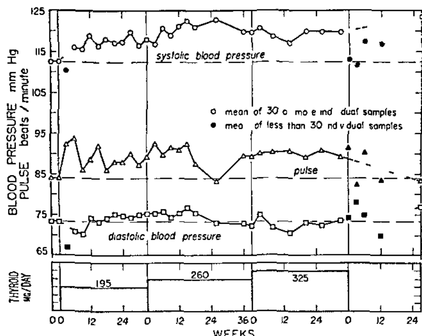


FIG 4 The effect of the long term administration of 3–5 grams of desiccated thyroid on blood pressure and pulse rate

virtually no thyroid hormone is present since endogenous production has been shut off if doses in excess of 3 grams were given) thus explaining the sharp rebound observed after 5 grams (see Figs 3 and 1) and 10 grams and lack of significant rebound after 3 grams

Serum total cholesterol concentrations show similar but less marked changes than the S_f^0 0–12 serum lipoprotein levels and a marked decrease in S_f^0 12–20 serum lipoproteins is again observed The response of S_f^0 20–100 and S_f^0 100–400 was again noted to be considerably less marked and more variable showing small decreases at the higher dosage levels only

Average weight loss was only 5 pounds and it is noteworthy that no

further weight loss occurred at the 5 grain level and indeed 8 patients regained some weight Figure 4 shows a very moderate mean increase in pulse rate from about 84 to 90 and a slight increase in mean systolic blood pressure from 113 to about 120, and no change in diastolic blood pressure Table I shows the highly significant drop in mean atherogenic index of 18 units observed in this study

TABLE I
EFFECT OF THYROID ON ATHEROGENIC INDEX VALUES (39 CASES)

Initial A I value	70 0 units
A I value at 36 wk on 5 grains thyroid	52 0 units
Change	—18 0 units

Next, I should like to present some data on the effect of intravenous *l* thyroxine This study was done by Strisower and co workers (9) Eight male and 8 female schizophrenic patients of comparable age dis tribution (mean age 37 years) received an intravenous dose of 6 mg of *l* thyroxine once a week for 16 weeks and a control group of 4 *patients received I V saline solution Of the initial group of 16 patients* 13 completed the entire course 2 were lost because of poor veins and 1 because of excessive weight loss Figure 5 shows a very sharp and pronounced drop in $S_f{}^0$ 0–12 serum lipoprotein concentrations and smaller but statistically highly significant ($P < 0\ 001$) decreases in $S_f{}^0$ 12–20 and total serum cholesterol levels No statistically significant effects occurred in the $S_f{}^0$ 20–100 and $S_f{}^0$ 100–400 serum lipoprotein classes though a tendency for moderate decreases in these levels was observed in patients with high initial $S_f{}^0$ 20–100 and $S_f{}^0$ 100–400 serum lipoprotein levels A mean weight loss of 13 pounds occurred in these patients

Figure 6 shows the lack of effect of *l* thyroxine on systolic blood pressure however there was a mean decrease of diastolic blood pressure of about 10 mm of mercury ($P < 0\ 001$) and a mean increase in pulse rate of 17 beats per minute ($P < 0\ 001$)

Figure 7 shows the effect of *l* thyroxine on the high density serum lipoprotein spectrum A large drop in the major high density serum lipoprotein species HDL_3 is of interest though small but statistically significant decreases were also observed in the HDL_1 and HDL classes

A relationship of considerable practical significance is shown in Table II *The extremely high correlation coefficients for the* $S_f{}^0$ 0–12 and $S_f{}^0$ 12–20 serum lipoprotein bands indicate very strongly that *l* thyroxine may be expected to have a greater hypolipoproteinemic effect (for these two serum lipoprotein classes) the higher the initial

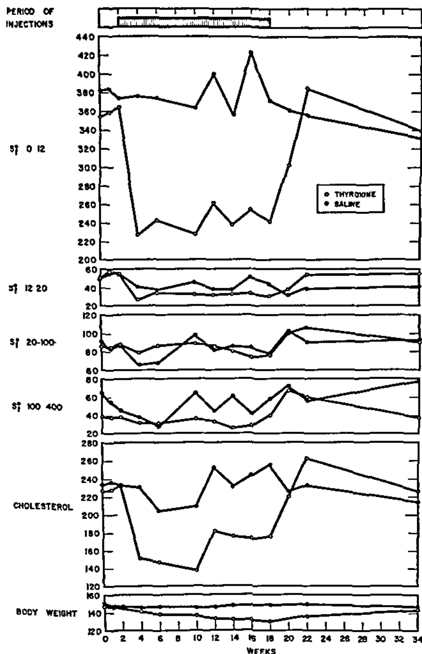


FIG 5 The hypolipoproteinemic effect of the weekly intravenous administration of 6 mg of l thyroxine

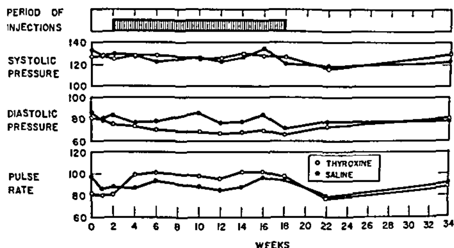


Fig 6 The effect of 6 mg per week of intravenous l thyroxine on blood pressure and pulse rate

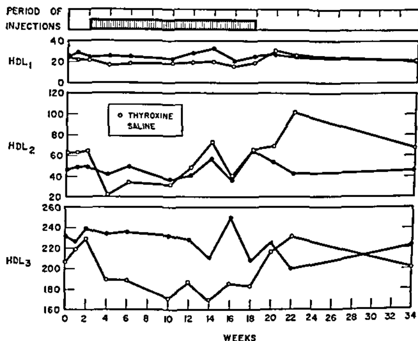


Fig 7 The high density hypolipoproteinemic effect of intravenous administration of 6 mg of l thyroxine per week

levels Thus patients with the greatest need for reduction of their $S_f{}^0$ 0–12 and $S_f{}^0$ 12–20 levels because of their high initial levels may be expected to respond with the largest reductions The correlation co efficients for the $S_f{}^0$ 20–100 and $S_f{}^0$ 100–400 serum lipoprotein classes are of only borderline statistical significance

TABLE II
RELATIONSHIP BETWEEN INITIAL LIPOPROTEIN CONCENTRATION AND MAGNITUDE OF HYPOLIPOPROTEINEMIC RESPONSE TO I V *l* THYROXINE
(6 mg week)

Lipoprotein class ($S_f{}^0$)	Correlation coefficient between initial concentrations and response to *l* thyroxine
0–12	+0 87
12–20	+0 98
20–100	+0 69
100–400	+0 60
0–400	+0 79

Next I should like to present some interesting data obtained in a recently completed study (12) employing *l* triiodothyronine Twelve male and 8 female schizophrenic patients (mean ages 37 and 42 re spectively) were given gradually increasing doses of *l* triiodothyronine for a period of 11 weeks until a dosage level of 200 μg /day was reached which was continued for an additional 11 weeks No patient in this group failed to complete the entire 22 week course because of hyper thyroid reactions though 4 patients did manifest minor reactions

By far the greatest and most consistent hypolipoproteinemic response was again observed in the $S_f{}^0$ 0–12 $S_f{}^0$ 12–20 and total serum cholesterol concentrations though smaller and statistically significant reductions of the $S_f{}^0$ 20–100 and $S_f{}^0$ 100–400 serum lipoprotein classes ($P < 0\,02$) occurred in most patients at the higher dosage levels studied (150–200 μg *l* triiodothyronine per day) It is worthy of note that in this experi ment the hypolipoproteinemic response of the female group of patients exceeded that of the male group by a significant amount Figures 8 and 9 show these effects graphically Protein bound iodine (PBI) levels tended to decrease and this decrease was again more marked in the fe male than in the male group Table III shows that the hypolipopro teinemic effect of *l* triiodothyronine was greater in the female group on an absolute as well as on a relative scale (reduction expressed as per cent of initial serum lipoprotein concentration)

Calculation of appropriate correlation coefficients showed again that initial $S_f{}^0$ 0–12, $S_f{}^0$ 12–20 and total serum cholesterol levels were sig

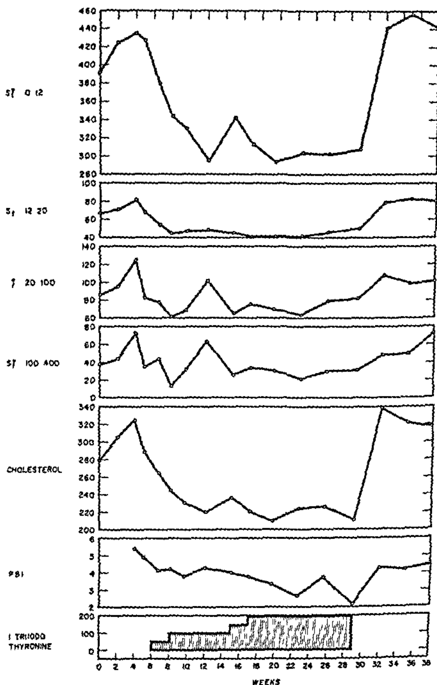


FIG 8 The effect on serum lipoproteins total serum cholesterol, and PBI of 50-200 μg per day of *l* triiodothyronine in a group of 8 female patients.

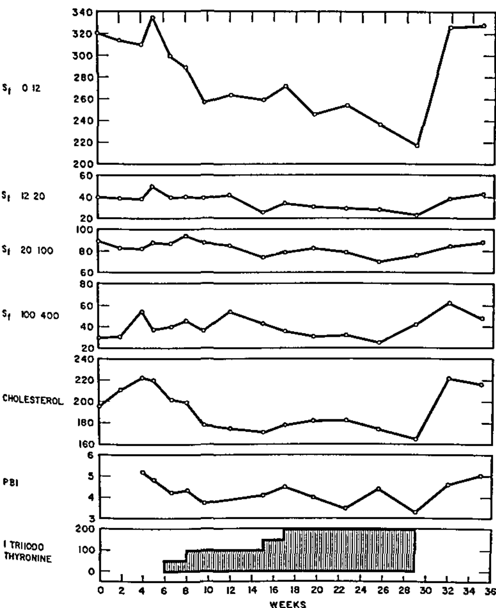


FIG 9 The effect on serum lipoproteins total serum cholesterol and PBI of 50–200 µg per day of *l* triiodothyronine in a group of 12 male patients

nificantly correlated with the degree of reduction in these levels brought about by the hormone in the female group of patients It is interesting to note that in the male group only the S_f^0 12–20 class was so correlated These relationships are shown in Table IV

Changes in mean body weight and mean pulse rate were quite moderate (reduced by 7 pounds and increased by 7 beats per minute

TABLE III
THE HYPOLIPOPROTEINEMIC EFFECT OF *l* TRIIODOTHYRONINE IN MALE AND FEMALE PATIENTS

	Sex	Pre[a] (mg / 100 ml serum)	Change (mg /100 ml)	Per cent change	Significance
S_f^0 0–12	M	318	− 73	−23	<0 001
	F	431	−127	−29	<0 001
S_f^0 12–20	M	41	− 13	−32	<0 001
	F	76	− 32	−42	<0 001
S_f^0 20–100	M	85	− 8	− 9	<0 02
	F	99	− 27	−27	<0 01
S_f^0 100–400	M	43	− 10	−23	NS
	F	51	− 22	−43	<0 02
Cholesterol	M	218	− 41	−19	<0 001
	F	311	− 92	−29	<0 001

[a] Mean pretreatment serum lipoprotein concentrations

TABLE IV
RELATIONSHIP BETWEEN INITIAL SERUM LIPOPROTEIN LEVELS AND MAGNITUDE OF HYPOLIPOPROTEINEMIC RESPONSE TO *l* TRIIODOTHYRONINE

	Sex	Pre[a] mg / 100 ml	Change mg /100 ml	RCC[b]	Significance
S_f^0 0–12	M	318	− 73	0 12	NS
	F	431	−127	0 83	≃0 01
S_f^0 12–20	M	41	− 13	0 60	<0 05
	F	76	− 32	0 85	<0 01
S_f^0 20–100	M	85	− 8	0 43	NS
	F	99	− 27	0 36	NS
S_f^0 100–400	M	43	− 10	0 17	NS
	F	51	− 22	0 39	NS
Cholesterol	M	218	− 41	0 12	NS
	F	311	− 92	0 83	≃0 01

[a] Mean pretreatment serum lipoprotein concentrations
[b] Rank correlation coefficient

respectively) A significant reduction in mean systolic (6 mm Hg) and mean diastolic (13 mm Hg) blood pressure was noted

It is of interest to compare the physiologic effects of desiccated thyroid *l* thyroxine and *l* triiodothyronine Table V shows that at the maximum dosage levels employed in our studies (200 µg per day of *l* triiodothyronine 5 grains per day of desiccated thyroid and 6 mg per week of *l* thyroxine given intravenously) all three thyroid active substances have a quantitatively remarkably similar hypolipoproteinemic

TABLE V
EQUIVALENT HYPOLIPOPROTEINEMIC DOSES OF *l* TRIIODOTHYRONINE *l* THYROXINE AND DESICCATED THYROID

Dose	S_f^0 0–20 Initial (mg/100 ml)	S_f^0 0–20 % Decrease	S_f^0 0–400 Initial (mg/100 ml)	S_f^0 0–400 % Decrease
Triiodothyronine (200 µg /day)	419	−31	551	−29
l Thyroxine (6 mg I V /week)	417	−35	542	−29
Thyroid (5 grains/day)	432	−25	554	−24

TABLE VI
EFFECT ON BLOOD PRESSURE OF THREE THYROID-ACTIVE SUBSTANCES

	Systolic blood pressure Initial (mm Hg)	Systolic blood pressure Per cent change	Diastolic blood pressure Initial (mm Hg)	Diastolic blood pressure Per cent change
Triiodothyronine (200 µg /day)	130	−5	76	−17[a]
l Thyroxine (6 mg I V /week)	125	0	78	−14
Thyroid (5 grains/day)	112	+7[a]	73	0

Represents statistically significant ($P < 0.01$) *differences* between figures marked [a] in each column

effect on the low density serum lipoprotein spectrum Table VI shows that *l* triiodothyronine has a marked hypotensive effect ($P < 0.01$) compared to 5 grains of desiccated thyroid an equivalent hypolipoproteinemic dose This suggests the use of *l* triiodothyronine when a moderate hypotensive effect is desired in addition to a profound hypolipoproteinemic effect Another point of difference is the reduction in PBI levels produced by high doses of *l* triiodothyronine whereas high doses of thyroxine have been shown to increase PBI concentrations (2) As mentioned previously we observed a pronounced reduction in the high density HDL_3 serum lipoprotein level produced by *l* thyroxine but we have not been able to show any such hypolipoproteinemic effect of

l triiodothyronine in our female group of patients Because of the surprising nature of this apparent qualitative difference in the effects of the two hormones further studies are needed to establish this point

In conclusion we might ask what important concepts emerge from the clinical studies I have summarized for you? In answer to this question I should like to point out that the thyroid active compounds we have studied appear to have a lipoprotein specific effect on the S_f^0 0–20 serum lipoprotein class Reduction of this class of serum lipoprotein concentrations is quantitatively very considerable occurs promptly and predictably and shows a very high correlation with initial S_f^0 0–20 levels None of these statements apply to the S_f^0 20–400 serum lipoprotein spectrum Furthermore the specific hypolipoproteinemic effect of thyroid active substances makes excellent physiologic sense when considered from the point of view of the highly specific hyperlipoproteinemia found in myxedematous patients Figure 10 taken from studies of Gofman and associates (5) shows clearly that the hyperlipoproteinemia in myxedema is S_f^0 0–20 specific the remaining low density serum lipoprotein spectrum being indistinguishable from that of normal matched controls

One would expect that thyroid substance may be of specific benefit in the treatment of xanthoma tendinosum a metabolic disturbance characterized biochemically by a massive increase in the concentrations of the S_f^0 0–12 and S_f^0 12–20 serum lipoprotein classes Studies of Gofman and associates (4) have indeed confirmed this expectation Table VII illustrates the marked hypolipoproteinemic effect of desiccated thyroid observed in a series of such patients

TABLE VII

S_f^0 0–20 HYPERLIPOPROTEINEMIA

EFFECT OF THYROID ON S_f^0 0–20 LEVELS

Case	Dose (grains)	Time (months)	Pre S_f^0 0–20 (mg / 100 ml)	On S_f^0 0–20 (mg / 100 ml)	Change in S_f^0 0–20 (mg / 100 ml)
58F	4	1 5	1063	736	−327
68F	4	1 5	1193	837	−356
22F	7	4	930	776	−154
40M	6	5	1121	804	−317
41F	8	4	1090	686	−404
48M	3	5	690	602	− 88
33F	7	4	1056	695	−361
11F	5	4	953	540	−413
5F	3	15	955	551	−404
49M	3	3 5	751	537	−214

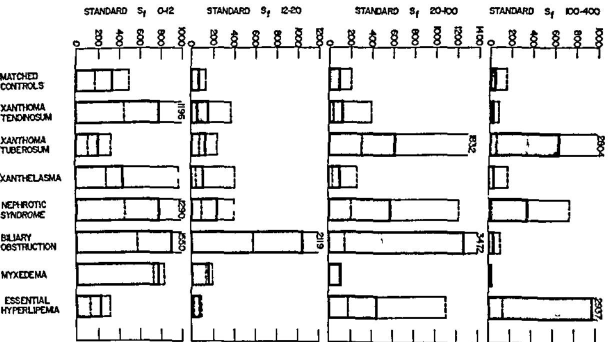


FIG 10 Mean serum lipoprotein levels (hatched areas) in various disease states Range of values is indicated by broken levels (or numbers) except for control group in which two standard deviations from the mean are so indicated

An understanding of the biochemical mechanism underlying the hypolipoproteinemic effect of thyroid active substances would most likely lead to further advances in the clinical use of these agents and deepen our understanding of certain broad aspects of lipid metabolism However nothing is known about this aspect of thyroid physiology In the absence of such information I should like to suggest a very simple tentative working hypothesis based on recent biochemical observations of several investigators Scaife and Migicovsky (8) have shown recently that liver homogenates prepared *from thyrotoxic rats showed a marked* depression (about 8 fold) of cholesterol synthesis from labeled acetate when compared to liver homogenates prepared from normal rats This finding suggests a decreased rate of hepatic cholesterogenesis from acetate but gives no further insight as to the site of action of thyroxine Such information was provided by Wolff and Ball (13) who found that thyroxine in concentrations as low as 1.5×10^{-6} inhibited malate oxidation by rat heart homogenates Further studies showed that thyroxine exerted its inhibition of the level of malic dehydrogenase and that addition of diphosphopyridine nucleotide (DPN) reversed this inhibition The relationship between DPN and thyroxine concentrations suggested to them a competitive interaction between DPN and thyroxine and subsequent studies (14) showed that the inhibition of purified malic dehydrogenase was competitive with respect to DPN To test whether thyroxine might inhibit other DPN linked *reducing systems* Wolff and Ball also investigated the effect of thyroxine on glutamate and α keto glutarate oxidation Both were found to be inhibited the degree of inhibition varying inversely *with the amount of DPN added Inhibition* of glutamate oxidation has been reported by Lardy and Feldott (7) and Wolff and Ball have cited evidence for the inhibition by thyroxine of crystalline yeast alcohol dehydrogenase liver *alcohol dehydrogenase* glutamic acid dehydrogenase and skeletal muscle lactic acid dehydrogenase These studies all indicate that there exists some evidence for an inhibitory effect of thyroxine on a variety of DPN linked dehydrogenases Since DPN is known to be required for the synthesis of cholesterol from acetate (1 3) it seems attractive to speculate that thyroid active substances exert their hypocholesterolemic effect by inhibiting one or more DPN linked dehydrogenase systems involved in the reductive biosynthesis of cholesterol from acetate The specific hypolipoproteinemic effect of thyroid substances may be due in part at least to the greater abundance and higher cholesterol content (weight per cent) of the S_f^{0} 0–20 serum lipoprotein class making this the most important cholesterol bearing species in the low density lipoprotein spectrum Decreased endogenous cholesterogenesis might therefore be

expected *a priori* to be a factor in the selective reduction of S_f^0 0–20 serum lipoprotein concentrations

REFERENCES

1 Bucher N L R and McGarrahan K *J Biol Chem* **222** 1 (1956)
2 Danowski T S Schwartz R Rhodes A J Yoder R D Punteren A J and Mateer F M *J Clin Endocrinol and Metabolism* **15** 1116 (1955)
3 Frantz I D Jr and Bucher N L R *J Biol Chem* **206** 471 (1954)
4 Gofman J W *Symposium of the Susan Baker Memorial Cardiovascular Foundation April 26 28* 1957
5 Gofman J W Rubin L McGinley J P and Jones H B *Am J Med* **17** 514 (1954)
6 Greer M A *New Engl J Med* **244** 385 (1951)
7 Lardy H A and Feldott G *Ann N Y Acad Sci* **54** 636 (1951)
8 Scaife J F and Migicovsky B B *Can J Biochem and Physiol* **35** 15 (1957)
9 Strisower B Elmlinger P Gofman J W and deLalla O *J Clin Endocrinol and Metabolism* in press
10 Strisower B Gofman J W Galioni E F Almada A A and Simon A *Metabolism Clin and Exptl* **3** 218 (1954)
11 Strisower B Gofman J W Galioni E F Rubinger J Pouteau J and Guzvich P *Lancet* i 120 (1957)
12 Strisower E H Gofman J W Strisower B and deLalla O *J Clin Endocrinol and Metabolism* **18** 721 (1958)
13 Wolff E C and Ball E G *J Biol Chem* **224** 1083 (1957)
14 Wolff J and Wolff E C *Federation Proc* **15** 387 (1956)

DISCUSSION

OLIVER A number of the effects that Dr Strisower has reported have been known for many years but the interesting point to me is the enormous dose which he has been administering Most of the changes which he has seen would be expected changes but the decrease in the concentration of high density lipoproteins following thyroid administration is unexpected We have administered thyroxine at the level of 600 µg to a group of hypercholesterolemic men with coronary disease this was followed by the expected fall in plasma cholesterol and β lipoprotein cholesterol but there was also a fall in α lipoprotein cholesterol We did not observe this fall in both lipoprotein fractions with smaller doses such as 300 µg Precisely the same situation holds with 3 5 3 *l* triiodothyronine depression of both these lipoprotein fractions occurred at the comparatively high dose level of 100 µg a day but at a level of 40 or even 60 µg this is not so At this lower dose level there is the usual reciprocal relationship with elevation of the α lipoprotein fraction I thought that this might be interesting in view of the fact that this dissociation is not very common and it appears that it might be a dose effect

STRISOWER I am very glad you have brought up these very interesting observations as they remind me to mention that when we studied the effect of *l* triiodothyronine on high density lipoproteins we noted that in the female group there was no effect at all whereas *l* thyroxine caused a marked decreased in the major high density serum lipoprotein component HDL_3 Certainly this requires further investigation as it represents one of the few qualitative differences between *l* thyroxine and triiodothyronine that we have been able to observe

BOYLE In a small series of 4 normal individuals which we gave from 5 to 7 grains of desiccated thyroid a day for a period of 4 months and kept in caloric balance without any weight loss we could not demonstrate these changes

STRISOWER This is an interesting point because we studied 4 normal individuals on 3 grains for a period of about 4 weeks and they all demonstrate the changes I discussed above However at that level of 3 grains there was an escape after 4 weeks which we concluded was due to the relatively small dose of desiccated thyroid used

FREEDBERG What was the PBI level in the patients given desiccated thyroid and thyroxine? Did they show a curve of increase early and then go down and to what level did they go down?

STRISOWER We have data on PBI levels only in the triiodothyronine study My comments on PBI levels following thyroxine administration refer to work done by Danowski who gave very large doses of thyroxine to euthyroid individuals and found a marked increase in the PBI level which then returns to normal levels about 10 days after cessation of thyroxine administration

ADLERSBERG I was wondering Dr Strisower whether you have any information concerning the effect of acetic acid or perhaps propionic acid analogs of triiodothyronine and thyroxine on serum lipoproteins and lipids

STRISOWER I am sorry to say that we do not

FURMAN We have some information Dr Adlersberg regarding the administration of triiodothyroacetic acid (Triac) in doses of 4 to 8 mg per day to a 49 year old woman in general good health Control serum lipid values over a 6 month period prior to the administration of Triac ranged between 191 and 228 mg % for cholesterol and 225 to 272 mg % for phospholipid Lipoproteins determined refractometrically showed a moderate preponderance of β over α or high density lipoproteins On the 4 mg daily dose the serum cholesterol level fell to 153 mg % phospholipid to 208 mg % and there was a prompt reversal in the α β lipoprotein ratio due to a reduction in β lipoprotein concentration without increase in α lipoprotein When the dose of Triac was increased to 8 mg per day there was a further reduction in serum cholesterol to 129 mg % and slight further decrease in phospholipid to 190 mg % There was no further significant change in the α β lipoprotein ratio We have not observed increase in α lipoprotein concentrations subsequent to the administration of thyroid active agents to euthyroid subjects as has been described by Oliver and Boyd

There was no evidence of escape during Triac administration in doses of 4 or 8 mg daily over a 110 day period to this euthyroid woman These doses were well tolerated although I would not say that she failed to show any evidence of hyperthyroid effect She had a slight increase in resting pulse rate and lost 3 kg of body weight but I think generally she tolerated this dose very well I can vouch for the 2 mg daily dose as an asymptomatic one and yet retaining a sustained hypocholesterolemic effect because both Dr Palmer Howard and I took Triac in this dosage for 4 months

STRISOWER May I ask Dr Oliver to comment on this with regard to his experience with triiodothyroacetic acid?

OLIVER With Triac we find that there is depression of the β lipoprotein cholesterol fraction with elevation of the α lipoprotein cholesterol at the 3–4 mg level

BOYD Dr Strisower in any objective measurement which you have made was there any real difference between desiccated thyroid *l* thyroxine or *l* triiodothyronine?

STRISOWER I will say this that at the dosage levels studied responses to these

substances were qualitatively similar with one exception and that was the diastolic blood pressure and the systolic blood pressure which went down with triiodothyronine went down somewhat less with *l* thyroxine and did not change or even increased with desiccated thyroid

HOWARD I would like you to repeat the changes in the systolic blood pressure in the patients who were getting desiccated thyroid and those receiving triiodothyronine As I recall the systolic pressure in the thyroid group went up from 112 to 119 whereas in the triiodothyronine group it went down from the pre treatment level of 135 to 128 Such divergent changes would not appear to be of much significance clinically especially since the two groups started with different blood pressure levels

STRISOWER I agree that the changes in systolic blood pressure occurring following triiodothyronine administration compared to those occurring with desiccated thyroid are not necessarily clinically significant In absolute terms these changes are relatively small though they can be shown to be statistically of significant consistency and magnitude The effect of the pretreatment blood pressure level on the direction of these effects is difficult to evaluate however I should like to point out that the pretreatment levels of diastolic blood pressure were quite comparable in these two studies and yet triiodothyronine produced a quite considerable reduction in diastolic pressure (statistically highly significant and probably of clinical significance also) compared to the lack of effect of desiccated thyroid

CHAPTER 24

The Effects of Sex Hormones on Serum Lipids and Lipoproteins[1]

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In a discussion on hormones and atherosclerosis it is important to consider the effects of these agents on the concentration and composition of the various serum lipoproteins Not only is all of the cholesterol phospholipid and triglyceride present in the serum transported as con stituents of lipoproteins (4) but also in atherosclerosis certain of these serum lipoproteins are generally present in abnormal concentrations (1 6) Furthermore certain hormones and their analogs produce marked changes in the concentration and composition of the serum lipo proteins and they may do so in the absence of any appreciable change in serum lipid concentration (5)

SERUM LIPOPROTEINS

The properties and methods of separation and determination of the serum lipoproteins have been described extensively in several recent reviews (4 10 11) Because of their high lipid content the lipoproteins are of lower density than the other serum proteins The densities of the various lipoproteins differ depending on their lipid content and use is made of this in several methods of lipoprotein separation (7 12) In Fig 1 is shown a hypothetical gradient density tube with a density at the top less than the solvent density of serum (1 006) and with the density progressively increasing to 1 21 at the bottom Were the ultra centrifugation of serum to be carried out in such a tube the lipoproteins would be arrayed in the tube with those of lowest density at the top and those of highest density near the bottom The lipoproteins may be divided into two major classes those of density less than 1 063 ($D < 1\ 063$) and those of density greater than 1 063 ($D > 1\ 063$) and these classes may be further subdivided as shown in Fig 1

The lipoproteins of $D < 1\ 063$ appear in the Cohn fraction I + III and all except the very lowest density group have the electrophoretic mobility of β_1 globulins (8) These lipoproteins are of large particle size with molecular weights of 2 000 000 and higher (10) They differ

[1] Supported in part by grants in aid from the National Heart Institute (H 2965) and G D Searle and Co

from each other primarily in their triglyceride content with those of lowest density and largest size containing the most triglyceride

The lipoproteins of $D > 1\ 063$ appear in Cohn fraction IV + V and they have the mobility of α_1 globulins They have molecular weights of about 200 000 (10) and they are of higher protein content than the $D < 1\ 063$ lipoproteins

In our early studies (13) lipoproteins were separated into two fractions by Cohn fractionation In subsequent studies preparative ultracentrifugation was employed using the method of Havel *et al* (7) In this method the solvent density of serum is adjusted by the addition of

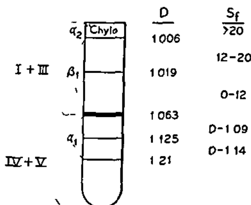


FIG 1 Distribution of serum lipoproteins after centrifugation in a gradient density tube Shown are the densities (*D*) Gofman flotation units (S_f) electrophoretic mobility and Cohn fractions

concentrated salt solutions the various fractions are then floated to the surface in a preparative ultracentrifuge and the fractions removed for analysis using a tube slicing device Generally both methods have yielded similar results However the method of preparative ultracentrifugation permits the separation of the lipoprotein fractions into subfractions and furthermore it permits their separation from other plasma proteins

THE EFFECTS OF ESTROGEN ADMINISTRATION

In the studies of Russ *et al* (13) it was found that there were marked differences in the lipoprotein patterns of young women as compared to young men and to older women Young women tended to have lower total serum cholesterol concentrations and this reduction *in serum cholestol was a reflection of* lowered cholesterol in fraction I + III The cholesterol in fraction IV + V + VI was slightly higher in the young women and the percentage of cholesterol in that

fraction was appreciably higher in young women than in any of the other groups

Because of these differences and because of the low incidence of coronary heart disease in young women study of the effects of the administration of sex hormones on serum lipoproteins was undertaken (14)

In Table I are summarized the changes observed after the administration of estrogen The subjects were male survivors of myocardial infarctions and they received either 1 mg of ethinyl estradiol per day or 15 mg of estrogenic substances USP (Premarin) per day The

TABLE I
EFFECTS OF ESTROGEN ADMINISTRATION IN MALE SURVIVORS OF MYOCARDIAL INFARCTION (3)

	Before	After
Plasma		
Cholesterol (mg/100 ml)	268	222
Cholesterol phospholipid	0 97	0 75
Fraction IV + V + VI		
Cholesterol (mg/100 ml)	31	58
Per cent of total cholesterol	12	28
Cholesterol phospholipid	0 46	0 44
Fraction I + III		
Cholesterol (mg/100 ml)	228	157
Per cent of total cholesterol	69	42
Cholesterol phospholipid	1 22	1 04

changes in serum lipids and lipoproteins were apparent within 1 week after the administration of estrogen and they became maximal within 6 weeks after estrogen therapy was commenced At the doses used there were marked decreases in serum cholesterol concentration with the mean decrease being 21% This fall in serum cholesterol was entirely within fraction I + III which decreased by more than 28% The cholesterol in fraction IV + V + VI increased appreciably and the percentage of cholesterol in alpha lipoproteins rose by almost 80% In these subjects the cholesterol phospholipid ratio fell in fraction I + III and this was reflected in the marked fall in the serum cholesterol phospholipid ratio When estrogen administration was discontinued the lipoprotein pattern returned to its original values within 1 or 2 weeks The pattern of changes could be repeatedly induced in a subject by readministration of estrogen to the subject

Changes similar to those seen following the administration of these estrogens have been seen after the administration of estradiol estrone

hexestrol and diethylstilbesterol (9 14) With all of these substances most of the patients experienced loss of libido impotence breast swelling and soreness and occasionally depression

Effects of Androgen Administration

The profound changes in serum lipoproteins induced by estrogen administration led to the study of the effects of androgen administration and the results are summarized in Table II Although the mean serum cholesterol increased after androgen administration in 11 of the 20 cases the total serum cholesterol did not increase or decreased very slightly The increase in the mean is due to the fact that in two cases

TABLE II
Effects of Administration of Methyltestosterone (3)

	Before	After
Plasma		
Cholesterol (mg /100 ml)	211	235
Cholesterol phospholipid	0 87	1 00
Fraction IV + V + VI		
Cholesterol (mg /100 ml)	46	28
Per cent of total cholesterol	22	14
Cholesterol phospholipid	0 46	0 43
Fraction I + III		
Cholesterol (mg /100 ml)	157	199
Per cent of total cholesterol	78	86
Cholesterol phospholipid	1 20	1.26

there were tremendous increases in serum cholesterol The median showed no change in serum cholesterol In all instances however cholesterol in fraction IV + V + VI decreased with the average decrease being 57% In all but three cases there was some increase in cholesterol in fraction I + III It is apparent that the most constant finding following the administration of androgen is the decrease in the cholesterol in the alpha lipoprotein fraction In most instances this is accompanied by increase in the cholesterol in fraction I + III At times this increase was sufficiently large to increase total serum cholesterol concentration No differences between the effects of methyltestosterone and parenteral testosterone propionate were found (14)

In an attempt to eliminate the feminizing effects of estrogens in the males a study (14) was made of combined estrogen and androgen administration It was found that despite administration of androgen in a dosage such that the lipid and lipoprotein effects of the estrogens would be reversed or eliminated the feminizing effects of the estrogen per

sisted Roughly 10 mg of methyltestosterone per day was equivalent to 1 mg of ethinyl estradiol per day in lipid effect but at this mixture feminization occurred regularly This is strikingly different from those effects seen in the chick These findings may explain why the chick is not a very satisfactory animal for the prediction of the efficacy of compounds on human serum lipoproteins

The Effects of the Administration of Synthetic Steroids

It has been our experience that the feminizing effects of estrogens when used at effective dosage is a serious disadvantage to the long term use of estrogens in patients with atherosclerosis For this reason considerable effort has been expended to find substances with which there might be greater dissociation of lipoprotein effects from feminizing effects All subjects used in these studies were males who had myocardial infarcts at least 2 months prior to the time of these studies

One compound 16α methyl 16 epiestriol 3 methyl ether was highly effective in altering the serum lipoprotein pattern but in the doses used it produced appreciable feminization Another compound 17α methyl 17 OH 5(10) estren-3 one [I] seemed promising on the basis of animal tests In animals it had some estrogenic some progestational and some androgenic effects (2) As can be seen in Table III it caused a slight

TABLE III
Percentage Change in Serum Lipid Distribution after Administration of 17α Methyl 17 OH 5(10) estren 3-one (Mean of Three Patients)

Amount per day (mg)	30 mg
Serum	
Cholesterol	+12 7
Phospholipid	+ 6 9
D < 1 063 (β)	
Cholesterol	+20 5
Phospholipid	+21 1
D > 1 063(α)	
Cholesterol	−48 6
Percentage of total cholesterol	−49 5
Phospholipid	−19 2

increase in serum cholesterol but considerable increases in beta lipoprotein cholesterol and considerable reduction in alpha lipoprotein cholesterol Clinically this compound did not have feminizing side effects however its effect in the human serum lipoproteins was that of an androgen

More recently we have studied the effects of the administration of

hexestrol and diethylstilbesterol (9 14) With all of these substances most of the patients experienced loss of libido impotence breast swelling and soreness and occasionally depression

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a 49 year old man to whom was administered [II] In this patient there was a gradual fall in total serum cholesterol and beta lipoprotein cholesterol a rise in cholesterol in alpha lipoprotein and a fall in the cholesterol phospholipid ratio In Fig 5 are shown the data on a 51 year old man to whom was administered [II] In this patient the serum

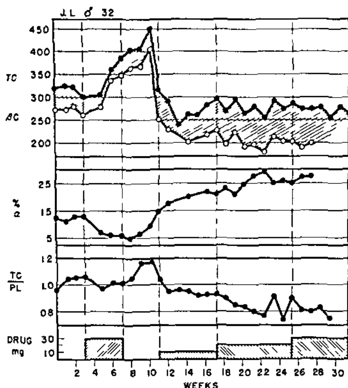


FIG 2 Effects of the administration of 17α methyl 17 OH 5(10) estren 3 one (I) in amounts of 30 mg per day and of 16α chlorestrone 3-methyl ether (II) in amounts of 10 20 and 30 mg per day TC = the total serum cholesterol concentration the shaded area represents cholesterol in the $D > 1\ 063$ fraction βC = the cholesterol in the $D < 1\ 063$ fraction % α = the percentage of total cholesterol in the $D > 1\ 063$ fraction TC/PL = cholesterol phospholipid ratio

cholesterol remained constant Nevertheless the percentage of cholesterol in alpha lipoprotein increased from 12% to 25%

In 2 out of 5 patients treated with [II] there was loss of libido and in one of them there was painful gynecomastia However when medication was discontinued all of the patients noted increase in libido

One other synthetic compound having appreciable estrogenic activity in rats is 2 methyl-3 ethyl-4 p anisyl Δ^3 cyclohexanecarboxylic acid

16α chlorestrone 3 methyl ether [II] The changes produced by this compound (Table IV) are seen primarily in the lipoprotein fractions and to a much lesser extent in the total serum lipids When these changes are expressed as percentages the change in the $D > 1\,063$ fraction is magnified since the lipid content of that fraction is low It is apparent that 20 mg per day of this compound is just about as effective as the larger dose

In Fig 2 are shown some of the changes induced by these steroids in a 32 year old male to whom was administered 30 mg of [I] In this patient there was a rise in total serum cholesterol a rise in beta lipoprotein cholesterol and a decrease in the percentage of cholesterol in

TABLE IV

PERCENTAGE CHANGE IN SERUM LIPID DISTRIBUTION AFTER ADMINISTRATION OF 16α CHLORESTRONE 3 METHYL ETHER (MEAN OF FIVE PATIENTS)

Amount per day (mg)	10 mg	20 mg	30 mg
Serum			
Cholesterol	− 6 5	− 3 9	− 7 1
Phospholipid	− 2 3	+ 3 0	+ 1 6
$D < 1\,063$ (β)			
Cholesterol	−15 2	−21 3	− 22 3
Phospholipid	*−14 0*	*−19 9*	—
$D > 1\,063$ (α)			
Cholesterol	+63 2	+87 0	+ 91 4
Percentage of total cholesterol	+66 1	+97 1	+111 0
Phospholipid	+27 5	+43 1	—

the alpha lipoprotein fraction The serum cholesterol phospholipid ratio also increased It was of interest that these effects persisted for 3 weeks after the administration of these compounds had been discontinued This patient was also given [II] and there was a significant drop in the beta lipoprotein cholesterol and there was an appreciable increase in the percentage of cholesterol in alpha lipoprotein and in the serum cholesterol phospholipid ratio In Fig 3 are shown the data on another patient to whom was administered [I] in the dosage of 30 mg per day In this patient there was no significant change in serum cholesterol The beta lipoprotein cholesterol rose while the alpha lipoprotein cholesterol decreased Following the administration of [II] cholesterol concentration decreased at first and there was an increase in the percentage of cholesterol in the $D > 1\,063$ fraction However during the period of administration of this compound and despite increased dosage the total serum cholesterol rose Notwithstanding this however the percentage of cholesterol in alpha remained high In Fig 4 are shown the data on

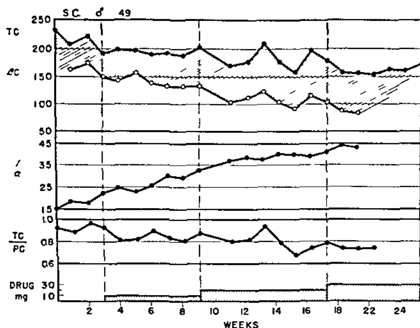


Fig 4 Effects of the administration of 16α-chlorestrone 3-methyl ether (II) in amounts of 10 20 and 30 mg per day See legend to Fig 2

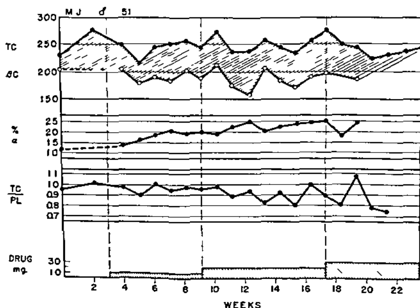


Fig 5 See legends to Figs 4 and 2

This compound has recently been administered in doses of 5 mg per day to 4 male survivors of myocardial infarctions In one of these patients there was a significant decrease in serum cholesterol Two of the patients but not the one showing the lipid changes had gynecomastia and one of those complained of insomnia and weakness

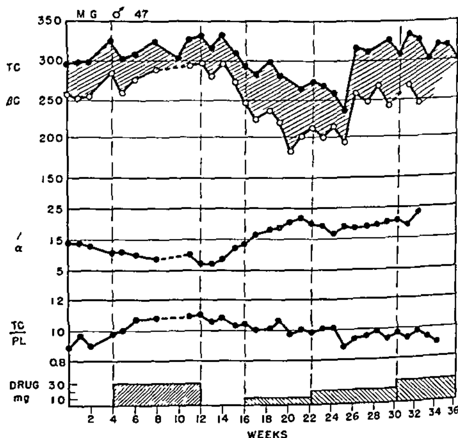


Fig 3 See legend to Fig 2

It is evident from these studies that there may be dissociation between the effects of these compounds on serum lipids and lipoproteins It may be that there is a sequence of changes with alterations in lipoproteins appearing first and subsequently changes in serum lipid concentrations Thus it might be expected that a very low dose of a potent compound would produce only the former changes but that increasing the dose may result in the latter changes as well However it may be that certain compounds affect only the lipoprotein distribution That this may be the case is suggested by the data on [II] which indicate

WG and CS and approximately 190 for cases AS and JW) marked differences in atherogenic index value (94 vs 117 and 128 vs 99) occur This is of considerable importance to the individual patient (and deserving of consideration by his physician) since the atherogenic index expresses his statistical risk of death from coronary disease the higher A I value in each of the two paired numbers representing an increase in this risk in excess of 100% Constancy of serum

TABLE A

PHYSICAL PROPERTIES OF SOME OF THE KNOWN HUMAN SERUM LIPOPROTEINS

Component	Hydrated density (g/ml)	Molecular weight	Molecular diameter[a] (Å)
HDL_3	1 145	170 000	78
HDL_2	1 075	360 000	101
HDL_1 (S_f)	1 050	$1\ 3 \times 10^6$	155
S_f4	1 041	$1\ 9 \times 10^6$	180
S_f6	1 035	$2\ 3 \times 10^6$	192
S_f10	1 021	$2\ 8 \times 10^6$	208
S_f13	1 015	$3\ 1 \times 10^6$	210
S_f27	0 99	$5\ 2 \times 10^6$	253
S_f40	0 96	9.2×10^6	309
S_f100	0 95	19×10^6	398
S_f400	0 93[b]	114×10^6	725
S_f1000	0 93[b]	460×10^6	1150 (0 115μ)
$S_f40\ 000$ (chylomicron)	0 93[b]	$1\ 2 \times 10^{11}$	7300 (0 73μ)

[a] For this calculation all lipoproteins are assumed to be spheres

[b] For these classes of lipoproteins the hydrated density has been estimated.

TABLE B

EFFECT ON ATHEROGENIC INDEX OF THE DISTRIBUTION OF SERUM CHOLESTEROL

Case	Cholesterol	Atherogenic index
CH	128	141
WG	229	94
AS	187	128
CS	231	117
JW	192	99
Normal 55-yr male	250	79

cholesterol levels in the presence of considerable variation in atherogenic index is due to the higher atherogenicity of the S_f 12–400 serum lipoproteins as defined by the expression A I $= 0\ 1 \times$ conc S_f^0 0–12 $+ 0\ 175 \times$ conc S_f^0 12–400 in addition to the higher cholesterol content of the S_f 0–12 class shown in Fig A

EDER We agree on the changes seen in the β lipoproteins after estrogen administration The disadvantage however of confining one s attention to the atherogenic index is that it omits any consideration of the α lipoproteins which change appreciably after estrogen administration

STRISOWER This is a very good point however in the extensive statistical analysis of cases of definite myocardial infarction as contained in the cooperative study no correlation between high density lipoproteins and a definite myocardial

that increasing doses do not produce any greater effects on the serum lipids

These findings introduce a new variable into our consideration of the clinical effectiveness of these compounds for we must now determine whether a compound which is effective only in altering lipoprotein distribution is effective in the prevention of atherosclerosis It is possible that compounds which are effective in lowering serum cholesterol, may be useful in the prevention of atherosclerosis merely by virtue of their ability to alter lipoprotein distribution

References

1 Barr D P Russ E M and Eder H A *Am J Med* 11 480 (1951)
2 Drill V A Personal communication (1957)
3 Eder H A *in* "Symposium on Atherosclerosis (I Page ed) National Academy of Sciences Washington 1954 p 228
4 Eder H A *Am J Med* 13 269 (1957)
5 Eder H A *Recent Progr in Hormone Research* 14 405 (1958)
6 Gofman J W Lindgren F T Elliott H A Mantz W Hewitt J Strisower B Herring V and Lyon T P *Science* 111 166 (1950)
7 Havel R J Eder H A and Bragdon J H *J Clin Invest* 34 1345 (1955)
8 Kunkel H G and Trautman R *J Clin Invest* 35 641 (1956)
9 Oliver M F and Boyd G S *Lancet* ii 1273 (1956)
10 Oncley J L *Harvey Lectures Ser* 50 71 (1956)
11 Oncley J L *in* Chemistry of Lipides as Related to Atherosclerosis (I Page ed) Charles C Thomas Springfield Illinois 1958 p 114
12 Oncley J L and Mannick V G *Abstr 126th Meeting Am Chem Soc* p 1 (1954)
13 Russ E M Eder H A and Barr D P *Am J Med* 11 468 (1951)
14 Russ E M Eder H A and Barr D P *Am J Med* 14 4 (1955)

Discussion

STRISOWER *Table A shows some of the physical properties of human serum* lipoproteins This table based on calculations of Dr Lindgren shows the increase in molecular weight from about 170 000 for the main high density component, HDL_3 to molecular weights of the order of 100 million and above for the lightest low density lipoproteins Associated with the increase in molecular weight is an increasing molecular diameter and a decrease in hydrated density

Figure A shows the increase in the weight per cent of lipid and decrease in protein content observed over the entire lipoprotein spectrum as one goes from the direction of high density to progressive lower density (and higher flotation rate) of lipoproteins The chemical composition shows a consistent increase in triglyceride and decrease in both free and esterified cholesterol content with increasing S_f^0 rate from 0 to 400 whereas the phospholipid content decreases only slightly The numbers *summarize more detailed quantitative information* on chemical composition which time does not permit us to discuss here

Table B shows the effect on atherogenic index of the distribution of serum cholesterol among the different serum lipoprotein classes With the total serum cholesterol level remaining essentially constant (*i e* about 230 mg% for cases

protein distribution have been those that have been described in the male It should be emphasized that finding young women with myocardial infarction has been in *our experience very difficult*

STAMLER The chairman might add that when one finds a few such menstruating women with clinical coronary heart disease they almost invariably manifest other abnormal findings e g hypertension or diabetes or hypercholesterolemia or kidney damage or combinations thereof

SAMUELS I would like to ask Dr Eder if he has studied the protein moiety at all I am not familiar with the protein structures of these lipoproteins but of course when we are studying the distribution of steroid hormones we find that the protein determines the distribution because of the secondary binding forces involved and the equilibrium thus established The estrogens apparently have altered the type of protein that is circulating and that can bind cholesterol and lipids I would like to know therefore whether he has studied the character of the protein moiety in volved and whether such moieties are present in excess of the bound lipid This would determine whether such proteins are playing a role positively or negatively with regard to the laying down of lipid

EDER The protein moiety is of course an essential constituent of the lipoprotein We have attempted to study it Thus we have measured the rate of incorporation of C^{14} labeled alanine in the protein of the lipoprotein in rabbits Others have done immunochemical tests on the separated lipoproteins and have also determined the amino acid end groups of the proteins We know of no studies in which any measurements of the lipoprotein proteins have been made following the administration of estrogen It should be emphasized that there is a great deal that we do not know about the protein moiety of the lipoproteins e g whether the rate of synthesis of this protein affects the concentration of lipids in the serum whether the rate of synthesis of the protein is dependent on the rate of lipid synthesis Another question is whether the protein can exist in the circulation free of lipid and able to bind lipid reversibly Such an "apoprotein" could not be detected by usual methods of lipoprotein separation since they are dependent upon the low density produced by the presence of lipids

In the studies on patients with atherosclerosis in which the serum lipoproteins were separated by Cohn fractionation it was found that those patients had larger amounts of proteins in fraction I + III in which β lipoprotein is found than did the normal It could be calculated that the increase in proteins was greater than could be accounted for by the increase in lipid Unfortunately Cohn fractionation does not separate all the various proteins in the fractions

FREEDBERG May I tell you about some preliminary experiments in which we tried to get some information on this question of whether the serum lipoproteins were unsaturated or could bind more cholesterol We (Kurland Lucas and Freedberg unpublished data) did the following in *vitro* experiments Free cholesterol was dissolved in Tween saline according to the method of Chaikoff *et al* at 37 C and incubated with plasma over a period of 1–2 hours We were able to show that there was a steady increase in the amount of cholesterol added to protein or precipitable with protein over this period of time until at 1 hour all the cholesterol was bound If one carried out this reaction at 6–10 C as opposed to 37 C this addition did not occur We have carried out similar experiments with plasma from a patient with hypothyroidism and hypercholesterolemia and we got exactly the same pattern or curve of binding The cholesterol serum level was of course much higher in the myxedematous patient then in our control

infarction could be demonstrated whereas there was a high correlation with the standard low density serum lipoprotein concentrations It is true that the high density lipoproteins do of course contribute some cholesterol but this is essentially a fixed quantity not correlated with the incidence of myocardial infarction

EDER It is my recollection that high density lipoproteins were not measured in the cooperative clinical study

STRISOWER We have measured high density lipoproteins at the Donner Laboratory in the cooperative and other studies but they were not published as part of the cooperative study report

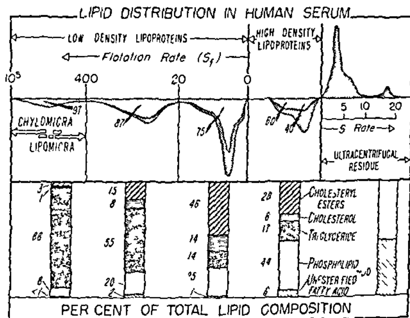


FIG A Lipid distribution in human serum

KATZ I think the record should show that the so called atherogenic index has semantic overtones which to the inexperienced carry a meaning not yet demonstrated I therefore propose that the index be called the Gofman or the Donner index This identifies it with the group purporting to ascribe meaning to it and leaves its significance for atherogenicity to some future time when all workers in the field are in accord that it has that significance

MARMORSTON It was a pleasure to listen to the erudite presentation of Dr Eder I am sure that this assembly is very much interested in at least 50% of our population namely the ladies and I think in this regard it would be of great interest to know what the experience is in treating women I would like to ask Dr Eder if he would be kind enough to tell us what his experience has been in treating women with estrogens

EDER We have had a very limited experience in treating females with estrogen We have been hesitant to do so because of the fact that we have used large doses of estrogen and have been concerned about the problem of producing uterine bleeding In the women that we did treat with estrogen the alterations in lipo-

Modification of the Effects of Adrenal Cortical Steroids and Androgens on Serum Lipids and Lipoproteins by Caloric Supplementation and by Isocaloric Substitution of Carbohydrate for Dietary Protein[1]

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During the course of a comprehensive study of the influence of endocrines on lipid metabolism and serum lipid transport in human subjects (5-9 16) and in animals (11 12 18) undertaken as an approach to the problem of the role of hormones in atherosclerosis we have become increasingly aware of the importance of nutritional and dietary factors as important determinants of the lipid and lipoprotein responses to the administration of hormonal substances

Recently we placed some of these dietary factors under scrutiny with rather interesting results in the first two areas of investigation We wish to submit to this Conference a report on work in progress in these studies which indicate the following (*1*) supplemental carbohydrate calories will prevent or abolish hypercholesterolemia resulting from adrenocorticosteroid administration (*2*) the isocaloric substitution of glucose for dietary protein results not only in alteration of serum lipids and/or lipoproteins but in a markedly altered lipoprotein response to androgen administration An additional finding of some interest is the absence of creatinuria following methyltestosterone administration in the absence of dietary protein

METHODS

Serum cholesterol was determined according to the Sperry and Webb modification (21) of the Schoenheimer Sperry method (15) and lipid phosphorus utilizing wet digestion according to Youngburg and Youngburg (22) and phosphate measurement according to the method of Fiske and Subba Row (4) The factor of 25 was used to convert "lipid phosphorus to phospholipid All blood was obtained in the post absorptive state Current and routine methods were employed for the several other serum urine and fecal analyses cited

For fractionation of serum into major lipoprotein classes aliquots of

[1] Aided by grants from the National Heart Institute National Institutes of Health, United States Public Health Service (H 2528 and H 2991) and the Oklahoma State Heart Association

WERTHESSEN I hope the chairman will forgive me if this comment runs the discussion period up a bit but it might save some discussion later Now the question I want to ask I am very concerned here about experimental design There is a paper which has just come out in *J Gerontol* (13 32 1958) written by L V Pilgeram showing that as he worked back toward the myocardial infarction the lipoprotein lipase co-factor decreased That is item 1 Item 2 if I gather the inferences of Dr Holman s data correctly just about everyone beyond the age of fifty is loaded with atherosclerosis My question therefore is in studies such as this shouldn t we have control data on people who do not have a myocardial infarct and let s find out *what happens to lipids in people who have atherosclerosis but who have not had a* coronary accident Finally in the use of the estrogens I would like to point out one potential danger A number of years ago we found an enzyme in the blood of people especially women which can convert estrone into non 17 ketosteroids Dr Axelrod in our laboratory has recently shown that in the bovine species estrone is converted into the inactive form of estradiol I use that term because some of the people are not acquainted with the chemical terminology Men also have this enzyme and I would like to suggest that in studies with estrogens one of the controls put in here be a determination in the blood of the enzyme which can alter these estrogens and do it drastically I would like to add here for some who have known of this work that Axelrod has finally cleaned up the problem completely with paper chromatograms and the fascinating thing to come out is that the substances produced under various conditions by various kinds of blood can be quite different and it is very possible that one of the complicating factors here is the presence or absence of this enzyme in the blood of the patients with whom we are working and I find it quite interesting in studies such as Dr Eder s that another man has shown that the system is altered by a myocardial infarct

EDER In our studies each patient is his own control since he is studied before during and after administration of an unknown compound so that it is not necessary to refer these changes to a control population Serum lipoproteins can change in the period immediately following myocardial infarction However the patients that have been used in these studies have been taken at least 2 months after a myocardial infarction

Metabolic balance techniques involving constant dietary intakes were employed in both studies The intake of calories was held at that level which maintained body weight during a preliminary "trial and error" period

TABLE I

MEAN PER CENT RECOVERY (± STANDARD ERROR) OF NATIVE SERUM CHOLESTEROL AND LIPID PHOSPHORUS AFTER ULTRACENTRIFUGATION AT 4 SOLVENT DENSITIES

Density	Per cent recovery	± S E	Number of samples[a]
	Lipid Phosphorus		
1 006	97 5	0 3	211
1 019	98 2	0 3	216
1 063	98 2	0 3	212
1.21	99 7	0 4	207
1.21 Supernatant	88 0	0 3	
1.21 Infranatant	11 7	0 3	
	Total cholesterol		
1 006	96 6	0 4	211
1 019	96 0	0 4	215
1 063	97 5	0 3	211
1.21	97 3	0 3	204
1.21 Supernatant	95 9	0 3	
1 21 Infranatant	1 4	0 1	

[a] Total number of serum samples each analyzed at least in duplicate obtained from 16 subjects over an 18-month period

In the second of the two studies which we will describe use was made of a formula diet in which 40% of the calories was provided by fat in the form of corn oil 15% by protein in the form of powdered skim milk and the remainder of the calories by glucose Multiple vita min capsules potassium iodide and ferrous sulfate were provided daily in constant amounts The formula diet was presumed to remain iso caloric when glucose was substituted for protein on a gram for gram basis During such periods of substitution the sodium potassium cal cium phosphorus magnesium and chloride content of the deleted protein was restored by the addition to the formula of a salt solution made up for that purpose

First let us consider the effects on serum cholesterol and phospholipid of adrenocorticosteroid administration to subjects on constant diets under metabolic balance conditions Our interest in the effects of adrenocorticosteroids on serum lipids and lipoproteins was stimulated by the thought that if "stress" does indeed cause hypercholesterolemia in some subjects the mechanism of action might be via a hypothalamic pituitary adrenocortical pathway Cortisone had already been reported

serum were pipetted into 4 cellulose centrifuge tubes and adjusted to solvent densities of 1 006 1 019 1 063, and 1 21 g /ml essentially according to the method of Havel, Eder, and Bragdon (14)

Centrifugation was carried out for 22 hours at 40 000 r p m (mean gravitational force 105 400 g) using the No 40 rotor in the Spinco Model L ultracentrifuge Following centrifugation the tubes were carefully removed from the rotor placed in a wire mesh test tube rack and lowered into a dry ice acetone bath (—58 to —62 C) and quick frozen The tubes and solid contents were sawed through at the middle of the opaque white zone separating the supernatant and infranatant fractions the lipid contents of which were readily revealed by their yellow color An electromagnetically driven jig saw with a fine toothed blade gave a clean smooth cut The frozen supernatant and infranatant fractions were then placed in individual funnels inserted in 100 ml volumetric flasks containing 20 ml methanol and allowed to thaw and drain with intermittent swirling Transfer of the fractions to the volumetric flasks was made complete by washing with dimethoxymethane The flasks contents were then made up to volume with dimethoxymethane shaken to insure adequate mixing and filtered Suitable aliquots of filtrate were taken for cholesterol and lipid phosphorus analysis and the solvent removed under a stream of nitrogen while in a 40 to 45 C water bath

The per cent recovery of native serum cholesterol and lipid phosphorus as represented by the sum of the super and infranatant lipid in each of the four tubes is shown in Table I Also tabulated is the per cent of native serum lipid found in the super and infranatant portions of the 1 21 g /ml density tube The latter indicate that virtually all of the serum cholesterol undergoes flotation (as lipoprotein) at this density while 10 to 12% of native serum lipid phosphorus fails to undergo flotation

[2] Lipoproteins undergoing flotation in a solvent density of 1 006 g /ml (which is approximately the density of human serum exclusive of proteins and lipoproteins) are chylomicrons and the very low density lipoproteins having flotation rates as determined by refractometric methods above S_f 20 (The designation S_f denotes a solvent density of 1 063 g /ml) In the solvent density of 1 019 g /ml additional lipoproteins of the S_f 12–20 flotation rate characteristic undergo flotation Additional lipoproteins of S_f 0–12 or —S_{1} $_{1}$25–40 ("beta) lipoprotein flotation rate characteristics undergo flotation in the solvent density of 1 063 g /ml while in the solvent density of 1 21 g /ml —S_{1} $_{1}$0–20 (alpha) lipoproteins also undergo flotation Virtually all of the cholesterol of native serum can be recovered in the "1 21 supernatant Approximately 10% of the lipid phosphorus of native serum fails to undergo flotation at this solvent density and only one third of this amount may be recovered from the infranatant by re extraction with petroleum ether (10)

The effects of adrenocorticosteroids on carbohydrate metabolism may be due in part at least to their ability to stimulate gluconeogenesis from protein (17 20) accounting for the negative balance and to inhibit the peripheral utilization of glucose (3) Sufficient impairment of glucose utilization and depletion of protein stores would eventually lead to enhanced mobilization of lipid (3) for energy purposes Under these circumstances extra carbohydrate calories by increasing the availability of glucose would lead to an increased secretion of insulin and reduced levels of plasma fatty acids (13) The possibility that administered insulin would have an effect similar to that of the extra carbohydrate calories in these subjects is now under study in our laboratory

Let us turn now to a consideration of the studies concerned with the protein free diets We were prompted to undertake these studies for two reasons First many conclusions relating serum lipid levels and mortality from heart disease have been derived from epidemiologic studies of population groups in which the diet is low in fat The important fact that such diets are almost always low in protein as well has often been ignored Second we were anxious to know if the ability of androgen to reduce high density alpha lipoprotein concentrations and alpha/beta lipoprotein ratios would be modified if the nitrogen retaining or anabolic property of the androgen were obtunded by eliminating dietary protein

The following study is representative of our experience An elderly woman with mild postmenopausal osteoporosis but otherwise in good health was placed on the corn oil skim milk glucose formula and the lipid and lipoprotein response to methyltestosterone administration recorded After isocaloric substitution of glucose for protein in the formula a second course of methyltestosterone was administered Serum and lipoprotein lipid values noted in this experiment are listed in Table III

Since only 40% of the calories in the formula were from fat the sole source of which was corn oil which is highly unsaturated (46 to 66% of total acids as linoleic acid) a reduction in serum lipid levels following the change from a conventional diet (period I) to the complete" formula was anticipated in view of the work of Kinsell Ahrens and others (2) When this subject was placed on the formula serum cholesterol and phospholipid fell to levels approximately 75% of those characterizing the conventional diet period i e from ranges of 198 to 209 mg % cholesterol and 290 to 295 mg % phospholipid to ranges of 140 to 153 and 185 to 195 mg % respectively The cholesterol and phospholipid content of the lipoprotein fractions fell proportionately

to cause hypercholesterolemia and hyperphospholipemia in animals and in man in certain instances (1)

So far we have administered 10 courses of adrenal steroids to 5 subjects under these conditions In 2 of these subjects marked increase in serum cholesterol and phospholipid levels regularly followed the administration of prednisone 30 mg/day (no changes in serum cholesterol or phospholipid levels were noted in the other 3 subjects)

When the lipid content of the serum lipoprotein fractions was determined it was noted that the rise in serum lipid levels following prednisone was associated with essentially proportionate increases in the cholesterol and phospholipid content of the alpha and beta lipoproteins in one subject while in the other the increase was slightly greater in the alpha fraction

The rise in serum cholesterol and phospholipid noted in these subjects could be prevented by adding 500 carbohydrate calories to the daily diet when prednisone was begun Alternatively the elevation in serum cholesterol and phospholipid levels could be abolished by the addition of these carbohydrate calories after the effect of prednisone had been established Under these circumstances slightly greater proportions of the serum cholesterol and phospholipid were found in the high density alpha lipoprotein fraction

In Table II are listed representative data from studies in one of these subjects a young woman with symptoms suggestive of anorexia nervosa of moderate degree

It should be pointed out that the 500 supplemental calories from carbohydrate delayed somewhat the negative nitrogen balance associated with the known catabolic properties of this steroid but did not prevent the negative nitrogen balance from developing

The administration of cortisone or prednisone to any of the 5 subjects studied to date has not resulted in any significant change in the degree of esterification of cholesterol or in the value for the cholesterol/phospholipid ratio in native serum or in either the lower density beta or the high density alpha lipoprotein fractions Hyperlipemia or lactescence of the serum has not occurred

The factor or factors responsible for the development of hypercholesterolemia and hyperphospholipemia in some subjects and not in others following adrenocorticosteroid administration are not clear Dose and period of administration do not appear critical factors for when hypercholesterolemia occurs it is noted promptly usually within 10 days On the other hand in 1 subject the prolonged administration of cortisone 75 mg/day for 2 months did not produce hypercholesterolemia

TABLE II (continued)

Day of study	Serum values mg %		Beta lipoprotein lipid				Alpha lipoprotein lipid			
	Cholesterol	Phospholipid	Cholesterol	Per cent of serum value	Phospholipid	Per cent of serum value[b]	Cholesterol	Per cent of serum value	Phospholipid	Per cent of serum value[b]
Pool of above 2	232	219	147	64 5	87	47 5	59	26 1	75	40 8
30	247	265								
34	228	245								
Pool of above 2	233	231	145	66 4	93	46 1	52	23 6	89	43 6
Mean	227	242	148	66 2	91	47 2	54	24 3	82	42 3
Standard error	3	4								
Period II										
44	260	273								
47	254	275								
Pool of above 2	263	251	176	66 8	109	51 6	65	24 6	79	37 5
50	298	305								
55	296	298								
Pool of above 2	296	287	203	68 0	128	53 7	74	24 9	88	36 8
57	289	293								
59	298	313								
Pool of above 2	300	291	203	67 8	115	46 5	70	23 3	107	43 0
Mean	284	287	194	67 5	117	50 6	70	24 3	91	39 1
Standard error	6	6								
Period III										
86	286	285								
89	271	278								
Pool of above 2	277	271	178	71 0	124	51 4	51	20 2	91	37 6
92	264	250								
96	272	270								

TABLE II[a]

EFFECTS OF PREDNISONE ON CHOLESTEROL AND PHOSPHOLIPID CONTENT OF NATIVE SERUM AND ALPHA AND BETA LIPOPROTEINS

(Subject OV Female Age 34)

	Serum values mg %		Beta lipoprotein lipid				Alpha lipoprotein lipid			
Day of study	Cholesterol	Phospholipid	Cholesterol	Per cent of serum value	Phospholipid	Per cent of serum value[b]	Cholesterol	Per cent of serum value	Phospholipid	Per cent of serum value[b]
Period I										
1	215									
19	219	238								
22	228	250								
Pool of above 3	221	233	151	67 8	94	47 9	51	23 1	83	42 5
26	219	248								
28	228	250								

[a] Explanatory notes

Period I — Control

Period II — Prednisone 30 mg /day Serum cholesterol and phospholipid concentrations significantly increased over Period I $P < 0.001$ for cholesterol and $P < 0.001$ for phospholipid

Period III — Control

Period IV — Prednisone 30 mg /day Serum cholesterol and phospholipid concentrations significantly increased over Period III $P < 0.01$ for cholesterol and $P < 0.001$ for phospholipid

Period V — Posttreatment controls Serum cholesterol and phospholipid significantly lower than Period IV $P < 0.001$ for cholesterol $P < 0.01$ for phospholipid

Period VI — Prednisone 30 mg /day plus 500 additional calories from carbohydrate Serum cholesterol and phospholipid levels show no significant change from Period V

Period VII — Prednisone continued 30 mg /day Extra carbohydrate calories withdrawn Serum cholesterol significantly ($P < 0.001$) higher than Period VI Phospholipid increase over Period VI is not statistically significant

Period VIII — Prednisone discontinued Serum cholesterol and phospholipid significantly lower than in Period VII $P < 0.01$ for cholesterol $P < 0.001$ for phospholipid

[b] Actually refers to per cent of lipid phosphorus found in 1 21 supernatant since approximately 10% of native serum lipid phosphorus fails to undergo flotation at this solvent density (10)

TABLE II (*continued*)

Day of study	Serum values mg %		Beta lipoprotein lipid				Alpha lipoprotein lipid			
	Cholesterol	Phospholipid	Cholesterol	Per cent of serum value	Phospholipid	Per cent of serum value[b]	Cholesterol	Per cent of serum value	Phospholipid	Per cent of serum value[b]
Period VI										
159	235									
161	243	273								
163	259	296	151	59.7	115	43.4	63	24.9	112	42.2
166	221	270								
168	279	315	169	61.3	134	47.1	71	25.8	110	38.6
169	266	328								
171	255	275	144	55.2	90	37.4	69	31.1	111	40.0
Mean	251	293	155	58.7	113	42.6	71	28.3	111	42.3
Standard error	7	13								
Period VII										
176	290	300								
181	335	329	226	66.7	137	47.6	93	27.5	124	42.9
183	307	318	210	64.9	135	47.6	89	27.7	121	42.5
187	323	320	212	65.8	141	48.1	89	27.6	123	41.9
Mean	314	317	216	65.8	138	47.8	90	27.6	123	42.4
Standard error	10	6								
Period VIII										
198	238	245								
201	245	250								
203	258	251	167	65.5	103	44.5	61	27.4	103	44.1
205	261	264	174	68.1	112	46.9	61	23.8	105	43.9
Mean	251	253	171	66.8	107	45.7	61	25.6	104	44.0
Standard error	5	4								

TABLE II (*continued*)

Day of study	Serum values mg %		Beta lipoprotein lipid				Alpha lipoprotein lipid			
	Choles terol	Phospho lipid	Choles terol	Per cent of serum value	Phospho-lipid	Per cent of serum value[b]	Choles terol	Per cent of serum value	Phospho lipid	Per cent of serum value[b]
Pool of above 2	280	252	193	71 3	115	51 9	59	21 7	87	38 8
99	270	273								
103	287	263								
Pool of above 2	275	266	189	71 8	117	51 4	58	21 9	90	39 3
Mean	276	268	187	71 4	119	51 6	56	21 3	89	38 6
Standard error	3	4								
Period IV										
110	284	283								
113	305	300								
117	348	338								
120	313	310								
Pool of above 2	315	320	212	69 2	127	45 6	75	24 5	127	45 8
125	295	296	202	69 5	133	51 4	74	25 4	102	39 4
Mean	315	313	207	69 3	130	48 5	75	24 9	115	42 6
Standard error	9	8								
Period V										
138	203									
140	258	268	187	69 6	115	45 3	65	24 3	117	46 0
142	230									
145	255	246	175	67 3	88	42 8	67	25 5	96	46 7
148	225									
153	285	267	207	71 8	120	49 6	60	20 9	102	42 1
Mean	243	260	190	69 6	108	45 9	64	23 6	105	44 9
Standard error	12	7								

TABLE III (*continued*)

Day of study	Serum values mg %		Beta lipoprotein lipid				Alpha lipoprotein lipid			
	Cholesterol	Phospholipid	Cholesterol	Per cent of serum value	Phospholipid	Per cent of serum value[b]	Cholesterol	Per cent of serum value	Phospholipid	Per cent of serum value[b]
C 10	176	238								
C 11	191	241	98	51 8	73	36 3	63	33 6	102	50 6
C 15	172	232	75	45 5	64	33 1	64	38 8	104	53 9
Mean	181	247	93	50 5	70	32 8	63	34 9	113	53 3
Standard error	4	7								
Period II										
4	148	208								
7	139	205								
9	140	190	59	44 4	47	28 7	62	46 8	103	63 7
11	140	186	59	43 3	47	29 7	68	50 5	103	65 5
15	150	203								
17	152	198	74	49 0	59	28 1	63	42 2	126	60 0
Mean	145	198	64	45 6	51	28 8	64	46 5	111	63 1
Standard error	2	4								
Period III										
24	110	155								
25	105	128								
28	103	130	75	73 8	52	42 2	19	18 7	57	46 7
30	104	145	71	71 4	55	46 5	21	20 7	51	42 6
Mean	105	139	73	72 6	53	44 3	20	19 7	54	44 7
Standard error	2	6								
Period IV										
37	147	168	88	63 1	61	44 7	43	31 0	67	49 1
39	136	173								
42	133	170	76	58 9	51	33 5	48	37 1	93	61 2

TABLE III[a]

EFFECT OF METHYLTESTOSTERONE ADMINISTRATION IN THE ABSENCE OF DIETARY PROTEIN ON THE CHOLESTEROL AND PHOSPHOLIPID CONTENT OF NATIVE SERUM AND ALPHA AND BETA LIPOPROTEINS (*Subject LR Female Age 70*)

	Serum values mg %		Beta lipoprotein lipid				Alpha lipoprotein lipid			
Day of study	Choles terol	Phospho-lipid	Choles terol	Per cent of serum value	Phospho-lipid	Per cent of serum value[b]	Choles terol	Per cent of serum value	Phospho lipid	Per cent of serum value[b]
Period I										
C 1	193	253								
C 3	177	228								
C-4	192	280	105	54 3	72	29 0	63	32 3	133	55 5
C 8	166	255								

[a] Explanatory notes

Period I — Control Subject on conventional diet

Period II — Subject on "complete formula Serum cholesterol and phospholipid values significantly lower than Period I $P < 0 001$ for both cholesterol and phospholipid

Period III — Methyltestosterone 50 mg /day administered while complete formula continued Further reduction in serum cholesterol and phospholipid values from Period II significant $P < 0 001$ for both cholesterol and phospholipid

Period IV — Methyltestosterone discontinued Increase in serum cholesterol and phospholipid over Period III significant $P < 0 001$ for cholesterol $P < 0 01$ for phospholipid

Period V — Isocaloric protein free formula Reduction in serum cholesterol and phospholipid from Period IV significant ($P < 0 01$) for cholesterol not significant for phospholipid Increase in proportion of serum cholesterol and phospholipid found in alpha fraction due undoubtedly in part to subsiding methyltestosterone effect

Period VI — Methyltestosterone 50 mg /day administered during protein free diet Further reduction in serum cholesterol and phospholipid from Period V significant $P < 0 001$ for cholesterol $P < 0 01$ for phospholipid

Period VII — Conventional diet no treatment Increase in serum cholesterol and phospholipid values over Period VI significant $P < 0 001$ for both cholesterol and phospholipid

[b] Actually refers to per cent of lipid phosphorus found in 1 21 supernatant since approximately 10% of native serum lipid phosphorus fails to undergo flotation at this solvent density (10)

i e there was no change in the relative distribution of these lipids among the major lipoproteins

The administration of methyltestosterone 50 mg per day for a 12 day period (III) produced the anticipated changes in lipoprotein lipid i e an increase in the cholesterol and phospholipid content of the beta lipoprotein fraction and a decrease in the alpha fraction both relatively and absolutely During this period native serum cholesterol and phospholipid values fell to levels approximately 50% of those characterizing the conventional diet period (I) Similar reductions in serum cholesterol and phospholipid levels following 17 methylnortestosterone administration to this subject (LR) while on a conventional diet have been observed in this laboratory previously (8)

Creatinuria was noted following methyltestosterone in the presence of dietary protein

Twelve days after methyltestosterone had been discontinued (period IV) glucose was substituted for protein in isocaloric amounts Over the ensuing 12 days (period V) serum cholesterol levels ranged somewhat lower than during period IV while serum phospholipid concentrations were slightly higher The major portion of the serum cholesterol and phospholipid could be found in the alpha lipoprotein fraction

A second course of methyltestosterone 50 mg /day was administered (period VI) This resulted in marked reduction in serum cholesterol and phospholipid to levels approximately 30 and 40% respectively of those values noted in period I These remarkable reductions in serum lipid values were associated with an entirely different lipoprotein response from that noted in period III when methyltestosterone was administered in the presence of dietary protein There was no increase in the beta lipoprotein cholesterol or phospholipid content during methyltestosterone administration in the absence of dietary protein and the major proportions of native serum cholesterol and phospholipid were found in the high density alpha lipoprotein fraction! It should be kept in mind of course that the marked reduction in native serum lipid values was associated with absolute decreases in the lipid content of both the alpha and beta lipoprotein fractions an increase in beta lipoprotein cholesterol and phospholipid content normally observed following testosterone administration failed to occur

In the absence of dietary protein methyltestosterone administration did not result in creatinuria

When methyltestosterone was discontinued after 8 days and the subject allowed to resume a conventional diet (period VII) serum cholesterol and phospholipid levels rose to those values characterizing period I of the study

TABLE III (*continued*)

Day of study	Serum values mg %		Beta lipoprotein lipid				Alpha lipoprotein lipid			
	Cholesterol	Phospholipid	Cholesterol	Per cent of serum value	Phospholipid	Per cent of serum value[b]	Cholesterol	Per cent of serum value	Phospholipid	Per cent of serum value[b]
Mean	139	170	82	61 0	56	39 1	45	34 1	80	55 1
Standard error	4	1								
Period V										
44	109	153	45	42 2	43	33 7	55	51 4	75	59 1
49	107	193								
51	117	173	44	38 8	33	22 9	63	54 9	103	71 0
54	123	180								
Mean	114	175	45	40 5	38	28 3	59	53 1	89	65 1
Standard error	4	8								
Period VI										
58	71	123	29	40 3	52	51 7	39	54 1	14	43 7
60	59	107	25	42 0	32	39 2	30	51 3	44	54 3
62	57	109	25	45 7	24	29 3	26	47 9	53	65 1
Mean	62	113	26	42 7	36	40 1	32	51 1	47	54 4
Standard error	4	5								
Period VII										
65	110	170[c]								
71	186	207	116	63 9	79	40 4	55	30 4	101	52 1
85	200	245								
92	193	252	99	55 8	81	36 2	65	36 6	126	55 9
106	214	260								
109	218	280								
113	198	243								
Mean	201	248	107	59 9	80	38 3	60	33 5	113	51 0
Standard error	5	10								

[c] Transitional values not included in mean

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REFERENCES

1 Adlersberg D *Am J Med* **23** 769 (1957)
2 Ahrens E H Jr *Am J Med* **23** 928 (1957)
3 Bierman E I Schwartz I L and Dale V P *Am J Physiol* **191** 359 (1957)
4 Fiske C H and Subba Row Y *J Biol Chem* **66** 375 (1925)
5 Furman R H and Howard R P *J Lab Clin Med* **46** 818 (1955)
6 Furman R H and Howard R P *Ann Internal Med* **47** 969 (1957)
7 Furman R H Howard R P and Norcia L N *Circulation* **16** 489 (1957)
8 Furman R H Howard R P Norcia L N and Keaty E C *Am J Med* **24** 80 (1958)
9 Furman R H Howard R P Shetlar M R Keaty E C and Imagawa R *Circulation* **17** 1076 (1958)
10 Furman R H Norcia L N Fryer A W and Wamack B S *J Lab Clin Med* **47** 730 (1956)
11 Furman R H Norcia L N Robinson C W and Gonzalez I E *Am J Physiol* **191** 561 (1957)
12 Gonzalez I E Conrad L L Norcia L N and Furman R H *Circulation* **14** 491 (1956)
13 Gordon R S Jr *J Clin Invest* **36** 810 (1957)
14 Havel R J Eder H A and Bragdon J H *J Clin Invest* **34** 1345 (1955)
15 Hawk P B Oser B L and Summerson W H Practical Physiological Chemistry 12th ed p 541 Blakiston Philadelphia 1947
16 Howard R P and Furman R H *Am J Med* **20** 950 (1956)
17 Long C N H Katzin B and Fry E G *Endocrinology* **26** 309 (1940)
18 Norcia L N Gonzalez I E Shetlar M R Peter J and Furman R H *Circulation* **14** 501 (1956)
19 Olson R E Vester J W Gursey D and Longman D *J Clin Invest* (*Abstr*) **36** 917 (1957)
20 Rosen F Roberts N R Budnick L E and Nichol C A *Science* **127** 287 (1958)
21 Sperry W M and Webb M *J Biol Chem* **187** 97 (1950)
22 Youngburg G E and Youngburg M V *J Lab Clin Med* **16** 158 (1930)

DISCUSSION

BOYD In your subject L R 70 in whom the fat carbohydrate and protein contents of the diet were altered I think the changes were made over 12 day periods and I wonder whether you thought this interval was long enough to stabilize the lipid levels before administering hormones? Also what is a mild anorexia nervosa?

FURMAN I will answer the second question first It means that I have seen

The relative amounts of cholesterol and phospholipid found in the two lowest density lipoprotein fractions were not significantly altered at any time during the course of the study

Studies in two additional subjects reveal similar patterns of response

It is worthy of note that studies currently underway in our laboratory indicate that the low serum cholesterol and phospholipid concentrations characteristic of the protein free formula diet alone are not dependent for their expression on the existence of a negative nitrogen balance Gradual restoration of protein to the diet, while the formula is kept isocaloric by removal of glucose has permitted the re establishment of a positive nitrogen balance without significant increase in serum cholesterol and phospholipid concentrations

The association of a low protein diet with reduced serum cholesterol levels has been reported (19) Not infrequently evidence of liver damage is present in these situations as manifest in the clinical syndrome Kwashiorkor or by reduced total to free cholesterol ratios or by bromsulfalein retention (2)

In none of the subjects studied by us has there been any reduction in the degree of esterification of the serum cholesterol nor have albumin and globulin levels been altered by the protein free diet Loss of body weight has been limited to 1 kg or less during the period of negative nitrogen balance induced by the protein free diet These subjects have remained clinically well and active about the ward

There are obviously a number of questions that will have to be answered in connection with these studies Is the nature or amount of fat in the formula critical? If the dietary protein in the formula were derived from sources other than milk would the responses observed be similar? What about individual amino acids? Is there a level of protein intake which is optimal" in relation to the nature and amount of fat and/or carbohydrate in the diet? What happens to serum lipids and lipoproteins when estrogen or other endocrines are administered in the absence of dietary protein?

We are bringing these questions under investigation as rapidly as possible There will be many other questions and there is need for much more investigation As Ahrens has said (2) "Our understanding of the effects of dietary protein on serum lipid levels is fragmentary"

The studies which we have described indicate that dietary alterations may alter serum lipids not only directly but indirectly as well by modifying the effects of endocrines on lipid metabolism and transport Both of these effects must be kept in mind as we study nutritional and lipid interrelationships

tion males I think it should be kept in mind that it has been reported that in hypercholesterolemic males who have suffered a myocardial infarction there is a tendency for the serum cholesterol levels to decline during the months following the infarct Hammarsten Cathey Redmond and Wolf *J Clin Invest* (*Abstr*) 36 897 1957) at the University of Oklahoma Medical Center are inclined to attribute this reduction in serum cholesterol levels during the several months following the infarct to the establishment of an improved doctor patient relationship and rapport and related psychosomatic factors Regardless of one s interpretation of this decline in serum cholesterol when seen following an infarct it is conceivable that the administration of cortisone to such individuals may appear to be associated with a fall in serum cholesterol which really has no direct relationship to the administered adrenocorticosteroid I don t say this with respect to the α β lipoprotein changes I accept these as steroid induced but I would like to throw out the question of whether the decline in serum cholesterol levels might have been observed had these individuals not been given adrenocorticosteroids

OLIVER I believe I can answer that question because these patients whom I mentioned just now all had a very abrupt and marked rebound in their lipid values in a matter of a week or 10 days following the cessation of ACTH or cortisone

STAMLER It is a matter for further work that after an infarct the cholesterol tends to decline This has certainly not been our experience observing a sizable number of people over several years There has not been any consistent change in cholesterol with time

FURMAN I should like to make one comment relative to Dr Oliver s last state ment and that is of course the sudden cessation of adrenocorticosteroids does not return the subject immediately to the same state as he was in before he began the steroids He is for several days and probably for weeks metabolically speaking, quite a different individual

STRISOWER I would like to ask a question of Dr Furman regarding his studies preceding the administration of the various cortical steroids Were these patients on an isocaloric diet so that when you gave them corn oil there was simultaneous limitation of other lipids in the diet?

FURMAN When the patients were placed on the formula containing corn oil the corn oil was the sole source of fat in the diet and the number of calories pro vided by the formula was kept constant throughout the study The calories provided were those which previous trial and error experience had indicated would maintain body weight

ZILVERSMIT I wonder if I may ask Dr Furman or any of the other endocrinol ogists present whether a pattern seems to emerge about the action of the corticoids on lipids It seems to me that we might think of corticoid action in terms of Seifter s terminology i e release of clearing factor inhibitor (CFI) and action on the liver to mobilize serum lipids It seems to me from what we have heard yesterday and this morning that this factor acts only on a liver that is depleted of glycogen In your studies on carbohydrate supplementation of the diet the reason why you don t get hyperlipemia after corticoids may be that the livers are rich in glycogen and the corticoid CFI sequence does not have this effect of lipid mobilization Similarly in some of our work on dogs we get no effect on serum lipids but when the dog is adrenalectomized and maintained on DCA (desoxycorticosterone acetate) and pre sumably the liver glycogen is depleted one or two doses of cortisone produce hyperlipemia

FURMAN I would certainly allow that such speculation is in order Dr

much worse cases Actually the young woman with mild anorexia nervosa was slightly depressed amenorrheic and a little underweight

The format which we employ in our metabolic balance studies is such that the fecal pool period is usually 6 days in length Urine is collected over 1 2 or 3-day periods depending on the particular needs of the situation In none of these studies have we waited less than 2 fecal pool periods or 12 days before making a change In most instances the serum cholesterol levels will plateau by the end of the second 6-day period so that only rarely has it been necessary to wait as long as 18 days In specific answer to your question in most instances the 12-day interval is sufficient to allow equilibrium to become established following some therapeutic or dietary manipulation

BOYD It is our experience that upon placing some subjects on a low fat diet (25 g/day) there is a rapid fall in the serum cholesterol during the first 2 to 3 weeks followed by a subsequent rise later producing final serum cholesterol values about 75% of the initial level

FURMAN We have had no experience with isocaloric substitution for fat The fat intake is constant in all of these studies but we have no evidence that study prolonged beyond 18 days would permit the re establishment of previous serum lipid levels

OLIVER I have been most interested in this communication and to see that there is an elevating action of prednisone in 2 out of these 5 cases and I would like just to mention some observations that we have made which appear to contradict this We have given ACTH or cortisone to 30 hypercholesterolemic subjects with coronary disease these were men who had a myocardial infarct a year or more earlier Twelve of these men received between 100 and 200 mg of ACTH daily and there was depression of the plasma cholesterol of the C/P ratio and of β lipoprotein cholesterol with elevation of the α lipoprotein cholesterol Cortisone administered from 50 to 200 mg daily to 18 of these men produced essentially the same changes Now this does not necessarily conflict with the observations of Dr Furman because it should be remembered that our men were reasonably healthy and engaged in full time employment They certainly had had a myocardial infarct but that was something in the past In contrast Dr Furman s 2 cases had anorexia nervosa or Sheehan s syndrome Earlier Dr Adlersberg showed that there was elevation of the plasma cholesterol and the β lipoprotein cholesterol in a group of patients who were ill with some collagen disease such as polyarteritis lupus erythematosus and rheumatic fever These patients had initially low serum cholesterol levels by the standards of this country and with the administration of cortisone or ACTH the ESR (erythrocyte sedimentation rate) and temperature came down the patients began to eat more their weight increased and the cholesterol levels rose So we may be dealing with two totally different groups of patients and our observations are not in contradiction to those of Dr Furman However in hypercholesterolemic men ACTH and cortisone appear to lower plasma cholesterol levels

FURMAN I agree with Dr Oliver with respect to his interpretation of the changes described in Dr Adlersberg s patients in the sense that they had sufficient improvement in their sense of well being as a result of adrenocorticosteroid or ACTH therapy to partake of more and more calories As a result their nutrition undoubtedly improved and under these circumstances one would expect the serum lipid levels to increase

With respect to the studies that Dr Oliver has presented which are concerned mainly if I understand correctly with hypercholesterolemic postmyocardial infarc

Dr Furman will find if he varies fat and protein in his diet on the cholesterol levels in his patients

FURMAN I want to point out that in this presentation we were careful to avoid the temptation to relate our findings to atherogenesis If I understand you correctly Dr Pick you indicate that a low protein diet is more conducive to atherogenesis in the chick than is a high protein diet with the same amount of dietary fat That of course is opposite to what one might be inclined to conclude from these studies We had begun to consider the possibility that the American diet was a luxus" diet as Dr Katz describes it not only from the point of view of its abundance of lipids but also from the point of view of its relative abundance of protein Maybe the chick and human are different!

STAMLER There seems to be an area of unclarity concerning the possible relationship between protein intake and atherogenesis in peoples like the Bantu Such peoples habitually ingest diets that compared to U S patterns tend to be low in total calories in total protein and animal protein in total fat and animal fat in refined carbohydrate in cholesterol Grains are the main nutrients foodstuffs of animal origin (meat poultry fish dairy products eggs) are minimally consumed Inevitably therefore the dietary pattern is a combination of low protein low fat low saturated fat low cholesterol Each of these dietary variables undoubtedly can be and has been correlated with the low incidence of atherosclerosis and atherosclerotic disease in these peoples The problem is which if any of these correlations is significant not only statistically but in a cause and effect way Within the context of these data this cannot be answered It is necessary to go beyond these data to other sources of information in order to arrive at valid conclusions

Let us reconsider the problem of possible interrelationships between protein intake and atherogenesis It has been suggested that since a positive correlation exists between level of protein intake and incidence of atherosclerotic disease high dietary protein is atherogenic low protein is antiatherogenic This hypothesis fails to reckon with two important sets of facts one socioeconomic the other animal experimental With respect to the first it has been amply demonstrated that with improvement of economic conditions increased per capita income the evolution of nutritional patterns is from grain diets to increasing intake of foods of animal origin As a result a concomitant increase occurs in consumption of total protein animal protein total fat animal (saturated) fat cholesterol Among the economically less developed peoples the field experiment possibly permitting a differential assessment i e the evolution to either a low protein high fat high saturated fat diet or to a high protein low fat diet practically never occurs for obvious reasons reflecting the nature of foodstuffs and their production Hence it is most difficult to assess which if any of these simultaneous changes may be decisively related in an etiologic sense to parallel trends indicating an increased incidence of atherosclerosis

Are there other data sources possibly elucidating this problem? Yes Considerable experimental evidence has been advanced This strongly suggests first of all that neither hypercholesterolemia nor atherogenesis can be induced merely by varying the level of protein intake in animals on diets low in fat and cholesterol Under these circumstances high protein intake is not atherogenic a cardinal fact demonstrated by Anitschkow almost 50 years ago Secondly experiments in chicks and rats demonstrate a definite effect of varying protein intake when the diet is concomitantly high in fat and cholesterol Under these circumstances high protein *inhibits* and low proteins *intensifies* hypercholesterolemia and atherogenesis (see Figs A and B) In a potentially atherogenic (high fat high cholesterol) diet there

Zilversmit but I want to point out that in these studies we have not encountered lactescence of the serum or hyperlipemia in the ordinary sense of the word The increments in lipids which we have observed seem to be limited to the serum cholesterol and phospholipoid components Perhaps if the period of prednisone administration had been several weeks instead of several days the serum might have become lactescent or hyperlipemic in the full sense of the word However these points do not negate your suggestions which I think are pertinent It should be recalled however that the administration of adrenocorticosteroids to animals with adrenal cortical insufficiency restores liver glycogen Furthermore work by Seifter and Baeder (*Proc Soc Exptl Biol Med* 95 747 1957) in rats whose livers were glycogen depleted indicated that these animals could handle the fat load resulting from lipid mobilizer induced elevations in plasma triglycerides as long as there was no exposure to hepatotoxic agents

ZILVERSMIT Do you feel that there is a possibility though that this is related to glycogen content and that all these events have a common denominator?

FURMAN Yes I believe there is a possibility of this

EDER I think that this technique of combining altered diets with hormone administration is very useful Were serum triglycerides or very low density lipoproteins measured in the patient who received diets high in carbohydrate? There has been considerable evidence to show that when fat is replaced by carbohydrate in the diet, serum triglyceride and low density lipoproteins increase in concentration I would wonder what the effect of corticoid administration in this situation would be I also should like to suggest that in the patient on the low protein diet who received methyl testosterone the failure of redistribution of cholesterol in the lipoprotein fractions could be due to the fact that the cholesterol in the fractions was already so reduced that there would be little opportunity for redistribution

FURMAN In answer to your first question Dr Eder we did not determine the triglyceride content of the four separated lipoprotein fractions We did determine however the cholesterol and phospholipid content of all four fractions and no significant changes in the cholesterol or phospholipid content of the low density lipoprotein fractions were noted Spot checks were made throughout these studies of the serum triglyceride concentration and there were no changes of consequence

With respect to your second question concerning the administration of methyl testosterone in the absence of dietary protein perhaps I did not emphasize enough that during this time there was an absolute reduction in the amount of cholesterol and phospholipid found in the high density or α lipoprotein fraction There was also an absolute reduction in the cholesterol and phospholipid found in the lower density or β lipoprotein fraction But the relationship of α to β lipoprotein remained either unchanged or a slightly greater proportion of the serum cholesterol and phospholipid was found in the α fraction This result was totally unexpected in view of what one ordinarily sees when testosterone is given in the presence of dietary protein

PICK I am particularly interested in the remarks about the decrease of protein levels in the diet with consequent decrease in hypercholesterolemia I am slightly hesitant to come back to atherogenesis and experiments in chicks but it might be of interest that we have some findings on the relationship of fat and cholesterol and protein in the diet and their influence on atherogenesis If we give a high protein diet with high fat and cholesterol hypercholesterolemia and atherogenesis in the animals are depressed If we decrease protein with the same amount of cholesterol and fat we get significantly enhanced atherogenesis I am very curious as to what

I was very interested in the effect of prednisone on serum cholesterol and serum phospholipids I showed yesterday that prednisone is more effective in the rabbit in this respect than cortisone and hydrocortisone It is very difficult to compare results in persons with normal circulating lipid levels with those obtained in patients who are really sick This was mentioned by Dr Oliver and later by Dr Furman In patients with such disorders as lupus leukemia and other severe general diseases these hormones have been administered in very large doses over periods of many weeks and months This is not strictly comparable to the short experiments performed in normal individuals

Finally a question about the liver Of course a protein free diet affects the function of the liver and this could modify the effect of testosterone on circulating lipids Even if you find no changes in the liver function tests there may be func

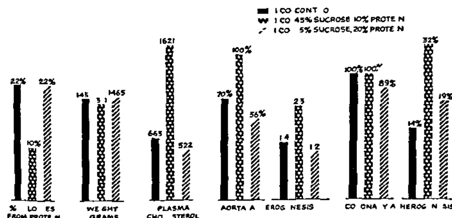


Fig B Effects of addition of refined carbohydrates to high fat high cholesterol chick mash—with and without reduction of dietary protein.

tional metabolic changes in the liver below the level which is detectable by these tests These could explain these observations

Furman Thank you Dr Adlersberg We find patients with hypogonadism useful for studies of this sort because they are particularly susceptible to the lipoprotein changes which one can induce with gonadal steroids and therefore are useful subjects for screening gonadal steroidal agents The young woman with anorexia nervosa had her endocrine parameters carefully evaluated There was evidence of diminution of ovarian function and decreased excretion of urinary gonadotropin In addition there was a slight degree of insulin hypersensitivity and slight hypoglycemia unresponsiveness Otherwise there were no observed abnormalities in endocrine function

I think your comment relative to the possibility that dietary protein depletion may alter liver function is a very good one It has occurred to us and this suggestion is also inherent in your remarks that the metabolic fate of administered steroids in the liver in the absence of dietary protein might be quite different from that which obtains when dietary protein is provided I would like to point out to the group and this is probably an unnecessary reminder that when we remove protein from the diet we dont really in effect, put the subject on a protein free

fore high protein intake is therefore antiatherogenic low protein intake is atherogenic

Based on these experimental findings it may be suggested that the Bantu exhibit low levels of serum cholesterol and low *occurrence rates of atherosclerotic disease* not because their protein intake is low but because their fat cholesterol intake is low It may be further hypothesized that sectors of the U S population ingesting diets high in total calories total fats saturated fats cholesterol refined carbohydrate and *low* (absolutely or relatively) in protein—a unique dietary aberration not uncommon among contemporary Americans particularly of lower income—may experience a compounding of the atherosclerosis problem because of low protein intake under these circumstances This is an aim for future clinical and epidemiologic research

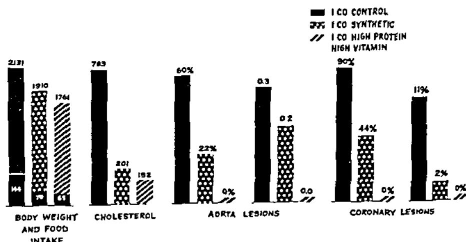


FIG A Effects of a high protein high vitamin diet in cholesterol fed cockerels

ADLERSBERG I would like to compliment Dr Furman and his group on this beautiful piece of work Of course those interested in metabolic disorders have known for many years that there are close interrelations between the metabolism of carbohydrates proteins and fats This work of Dr Furman s fits well into the present interest in the relation between carbohydrate and lipid metabolism It was mentioned yesterday that Albrink and Man found that addition of glucose to a simple fat loading meal affects the course of alimentary lipemia One may mention briefly the studies of Dole and Gordon on the relation between carbohydrate metabolism and the metabolism of nonesterified fatty acids (NEFA) showing a relationship between these two metabolic parameters

Now I am a little bit concerned about the selection of patients that Dr Furman used for his experiment Personally I don t trust a patient with anorexia nervosa even if she is a very mild case Also I would prefer for such a study patients in endocrinological equilibrium instead of a patient with Klinefelter s syndrome But I must add on the other side that each patient served as his own control and probably this criticism is not too valid under these circumstances But still I would like to see these time consuming experiments performed on more normal persons realizing fully how difficult it is to get them

CHAPTER 26

Prediction of Estrogenic Side Effects of Steroids in Man

VICTOR A DRILL RICHARD A EDGREN AND DONALD L COOK

Division of Biological Research G D Searle & Co Chicago Illinois

The effects of the standard estrogens on blood lipids in man have been the subject of fairly extensive study and literature in this area has been reviewed in other papers at this conference One approach to the study of atherosclerosis has been the preparation of new steroid compounds that possess the ability to alter blood lipids in the same direction as the standard estrogens (2) Such steroids possess a relatively low degree of estrogenicity when compared with estrone as a standard so that from the experimental view these compounds may be said to possess a higher lipid/estrogenic ratio than estrone or ethinyl estradiol For example SC 6924 (Manvene) has approximately 31% of the lipid shifting effects of estrone but it has only 1 6% the estrogenic activity of estrone or 0 2% the estrogenic effect of ethinyl estradiol Although such a compound can be called a weak estrogen one must be careful in predicting possible estrogenic effects in man particularly taking into consideration factors such as dosage which will influence the estrogenic response In view of the clinical trials of such steroids it is of interest to explain in more detail how such calculations and predictions can be made and applied to man

CALCULATION OF RELATIVE ESTROGENICITY

An example of how the low estrogenic potency of Manvene can be translated into clinical terms follows

If ethinyl estradiol = 100% (assigned value)
then Manvene = 0 2% estrogenic potency

(1) 10 mg Manvene ≎ 0 02 mg ethinyl estradiol (in estrogenicity)

(2) Ethinyl estradiol menopausal dose = 0 02–0 05 mg 1–3 times a day

(3) 10 mg Manvene/day is within menopausal dose range

It is obvious then that the administration of 10 mg of Manvene per day would be expected to produce an estrogenic response in man Robinson *et al* (3 4) and Davis (1) observed that doses of 5 or 10 mg of Manvene administered orally per day were estrogenic in male patients In these studies the estrogenic effects produced appear to be somewhat less than those observed with the standard estrogens Nevertheless the

regimen because he begins to eat his own body protein under these circumstances

STRISOWER With regard to the effect of diet on serum lipoprotein concentrations it is pertinent to summarize briefly the findings of Dr Nichols of the Donner Laboratory He studied 5 normal male individuals for periods up to 2 years on a rigorously controlled diet table on which the intake of animal fat vegetable fat, and carbohydrate were varied with protein varying only between fairly narrow limits Without going into detail this carefully controlled study showed that a high carbohydrate intake (high carbohydrate low fat diet) causes a specific increase in the S_f^0 20–400 serum lipoprotein class whereas saturated animal fats raise the S_f^0 0–20 serum lipoprotein concentrations The studies did not support the concept that unsaturated fatty acids per se have any effect on serum lipoprotein distributions The hypocholesterolemic effect of unsaturated fatty acids reported in most studies published to date appears to be the result of simultaneous reduction in the intake of saturated fatty acids and/or of carbohydrate required by the isocaloric nature of these investigations

HOWARD I would like to make a comment about the selection of the patients They were those who were available for hospitalization from an endocrine clinic and were suitable for long term metabolic balance studies It would be preferable sometimes to use patients who were more normal endocrinologically However the comment of Dr Adlersberg that we had been able to use the patients as their own controls is an important one Secondly Dr Boyd raised a question about the advisability of making changes in the management at such short intervals as 10 to 12 days As Dr Furman mentioned in describing the chart of lipid and lipoprotein data on subject L R 12 days on a new regimen proved long enough for the appearance of marked and significant changes almost without exception However the gradually increasing serum cholesterol values in the α fraction in Period V (see Table II) were attributable to the withdrawal of methyltestosterone in Period IV Thus in this instance 12 days for Period IV proved too short for the development of maximal effects although the significance of the changes and the interpretations remain clear

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be projected thus decreasing the total amount of compound administered and thereby the expected incidence of estrogenic side effects

Individual Variability

The response of a given population will vary over certain limits when a fixed dose is administered For example Shorr (5) has shown that the dose of ethinyl estradiol required to produce an estrous vaginal smear in the human subject can vary from 0 05 to 0 45 mg per day orally Thus when an "average" dose of a new steroid is administered and estrogenic effects measured the response may vary from absence of side effects to marked side effects in a given population

Oral/Parenteral Ratio

If the oral/parenteral ratios of new compounds differ significantly from 1 this factor must also be taken into consideration in calculating the dose to be administered and the estrogenic response to be expected

Slope of Dose Response Curves

The slope of the dose response curve for steroids that affect blood lipids is also important in predicting estrogenicity in man However at the present time this factor is difficult to evaluate quantitatively as it is not known how such slope relationships apply to man An example of this relationship is given in Fig 1

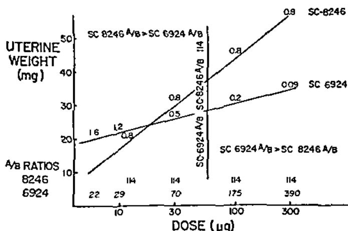


Fig 1 Effect of slope of estrogenic dose response curve on lipid/estrogenic (A/B) ratios of SC 8246 and SC-6924 Least squares regression lines of uterine weight against log dose are shown with potencies relative to estrone indicated above each line A/B ratios for each compound are shown at points at which relative estrogenic potencies are indicated Vertical line at a dose of 57 μg indicates point at which A/B ratios are identical for each compound

compound produces some estrogenic side effects in most patients Thus the interpretation of the experimental results and the clinical findings are in agreement

The estrogenic potency of Manvene (SC 6924) may also be compared with other standard estrogens (Table I) In Table I the equivalent estrogenicity of 10 mg of Manvene is compared with the daily oral menopausal dose of known estrogens When comparison is made with ethinyl estradiol and estriol one would predict that the daily administration of 10 mg of Manvene would produce estrogenic effects

TABLE I

PRODUCTION OF ESTROGENICITY IN MAN BY COMPARISON OF EQUIVALENT ESTROGENICITY AND MENOPAUSAL DOSE

Estrogenic potency		Equivalent estrogenicity 10 mg	Daily oral menopausal	O/P
Standard compound	Manvene (%)	Manvene (mg)	dose standard (mg)	Ratio standard
Ethinyl estradiol (100%)	0 2	0 02	0 02–0 15	ca 1
Estradiol (100%)	0 43	0 04	0 9 –1 8[a] (≈ 0 09–0 18)[b]	> 10
Estriol (100%)	13 0	1 3	0 12–0 48	ca 1
Estrone (100%)	1 6	0 16	3 (≈ 0 3)[b]	> 10
Premarin (100%)	4 8	0 48	1 25[c]	ca 1

[a] Maintenance dose
[b] Result when divided by O/P ratio
[c] As Na estrone SO_4 (≈ 0 92 mg steroid)

Estradiol and estrone are poorly absorbed when administered orally the oral/parenteral ratio being greater than 10 Thus if we divide the daily oral menopausal dose by 10 the theoretical amount absorbed is quite close to the estrogenicity expected of 10 mg of Manvene per day The oral/parenteral ratio may be closer to 15 and if division is made by this number the results are in closer agreement indicating that estrogenic side effects are to be expected when 10 mg of Manvene is administered orally each day

LIPID POTENCY

The sample calculations shown are for a given dose of compound and must be corrected for differences in lipid shifting potency If the lipid effect is low more of the compound will have to be given to affect blood lipids which will result in a relative increase in estrogenic activity Conversely if lipid shifting potency is high a smaller dose can

a complete estrogenic response in the female Thus estrogenicity will have to be decreased further to allow for such a factor Even minimal estrogenicity may be significant for when such steroids are administered over a period of weeks or months to man an accumulative endocrinological effect may be expected to produce side effects

Summary

Lipid shifting steroids with low estrogenic potency have been developed However the dose administered to man must be considered in predicting the occurrence of estrogenic side effects Sample calculations are presented and the data discussed in the light of compounds now available

References

1 Davis F W Jr Scarborough W R Mason R E Singewald M L and Baker B M *Am J Med Sci* 235 50 (1958)
2 Drill V A Cook D L and Edgren R A this volume Chapter 19
3 Robinson R W Cohen W D and Higano N *Circulation* 14 489 (1956)
4 Cohen W D Higano N and Robinson R W this volume Chapter 31
5 Shorr E *J Mt Sinai Hosp N Y* 12 667 (1945)

As reported in an earlier paper at this symposium SC 8246 has estrogenic effects approximately 0 8% those of estrone Inasmuch as this dose response curve is parallel to that of estrone the per cent estrogenicity remains the same at all dose levels (Fig 1) The curve for the lipid effect of this compound is also parallel to that of estrone Therefore as shown in Fig 1 the lipid/estrogenic (A/B) ratio remains at 114 for all dose levels The situation is somewhat different for SC 6924 (Manvene) Since the dose response curve does not parallel that for estrone the relative estrogenicity for this compound decreases from 1 6 to 0 09% as the dose is increased Thus the lipid/estrogenic ratio (A/B) increases from 22 at low dose levels to 390 at high dose levels The vertical line at a dose of 57 μg indicates the point at which the A/B ratios are identical for each compound Therefore to the left of this point SC 8246 has the more favorable ratio whereas to the right of this dose SC 6924 has the better ratio Clinically we are not sure whether we are working to the right or to the left of such a vertical line so that it is difficult to compare the two compounds and state on the basis of comparing nonparallel lines that one compound will have a significant advantage over the other We would suspect that we are working somewhat to the left of the vertical line but this is only supposition at the present time

Discussion

In view of the clinical trials on the effect of new steroids on blood lipids that are being undertaken it was thought of interest to explain how the possible estrogenic effects of such compounds may be predicted in man This is important for although a given steroid has considerably lower estrogenic potency when compared with standard estrogens such data must be interpreted in light of the dose to be administered to patients When one examines the experimental and clinical studies completed to date with SC 6924 and SC 8246 it would appear that each compound would be expected to be estrogenic in man at the lipid shifting dose administered Such has been found to be the case so that the results can be considered in complete agreement

It would follow that in order to decrease estrogenic side effects in man steroids with a better lipid/estrogenic ratio must be developed This is not simple to achieve for the minimal effective dose of standard estrogens needed to produce a lipid shift in man is about 5 to 10 times the menopausal dose of the compound Thus one must immediately have a ratio of 5 1 or 10 1 in a new compound in order to obtain the relative menopausal dose level of estrogenicity Further we should recognize that the menopausal dose level is usually sufficient to produce

Approaches to the Problem of the Relation of Emotions to Hormonal Function and Atherosclerosis

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Recently we at the Michael Reese Hospital have been tempted to go into the field of emotions in relation to atherosclerosis and hyper tension We have talked with a number of people and scanned the literature on this subject trying to orient ourselves So far all we have are bits of information A complete picture has not emerged Not enough facts are known to start a major study

First of all emotion should be clearly defined It is a term used psychologically and physiologically According to Websters dictionary it is a departure from the normal calm state of an organism of such a nature as to include strong feeling an impulse toward open action and certain internal physical reactions It may be any one of the states designated as fear anger disgust grief joy surprise yearning etc

The role of emotions in chronic diseases probably is of great im portance Emotions act on the body through the nervous system and via the hormones Contrariwise it has become clear recently that the nervous and endocrine systems—under the influence of internal as well as external environmental stimuli—may alter emotions moods neuroses and psychoses More important since chronic diseases may be affected by the nervous system and hormones they may be subject to the influence of emotions This could apply to atherosclerosis and hypertension as well as other chronic diseases

Studies on the relationship between emotions and chronic disease may involve an analysis of the somatic manifestations of emotional states in health and disease in order to classify the soma in psychosomatic It may involve the analysis of the effect of long standing disease on the emotional state of the patient Both of these aspects are however ir relevant to the study of the pathogenesis of atherosclerosis A more direct approach may involve an analysis of the role of emotions as acute triggers in making the manifestations of atherosclerosis appear This could be approached by unravelling their effects on (*1*) tissue metabo lism of proteins carbohydrates and lipids (*2*) blood coagulability (*3*)

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of deconditioning on these responses? How easy is it to induce these vascular responses and to abolish them relative to other types of responses?

6 Do intermittent hypertensive episodes lead to persistent hyper tension and vascular changes?

7 Do intermittent alterations in lipid metabolism lead to athero sclerotic vascular disease in the absence and in the presence of an atherogenetic diet?

8 Do intermittent alterations in the clotting mechanism predispose to thrombosis and to progressive changes in atherosclerotic plaques leading to clinically manifest disease and death?

9 Is it worth while to organize such studies on an interdisciplinary basis in which the several disciplines which could contribute to the analysis are represented and operate as a single team? Is it worth while to break down the barriers between the psychiatrist the internist and the investigator? Is it profitable to include the several basic areas of science and the several subdivisions of medicine in such a cooperative enterprise?

10 Finally in the present nebulous state of knowledge in this area, do we have enough facts to make it profitable for any serious group to undertake a major study?

The Chicago Heart Association has established a committee on Emotions and Cardiovascular Disease We have met a number of times and set as our immediate target for the next 2 years the exploration of the field through a series of conferences among ourselves We have representatives of the various disciplines meeting together so as to get to understand each other the scope of the problem and the possibilities of solving it We may from time to time invite someone outside of Chicago to meet with us for an exchange of views Only after this 2 year period will we consider whether to undertake a major study Whether we will decide in the affirmative or in the negative we do not know

Finally we would like to leave you with this thought—too much has been said on this subject on too little evidence We should do more research and less talking!

Discussion

BOYLE I would like to thank Dr Katz for what appears to me to be the soundest and most reasonable and sensible approach to this problem that I have been presented with heretofore Congratulations! I would have a question to ask which actually the speaker may or may not care to answer or attempt to answer because it is an unfair one In emotional stress such as that I am under at present with tachycardia and sweaty palms and not knowing whether it is due to the altitude here or to being before this austere group we look at the emotional responses in average people In some similar emotional traumatic episodes we will see in smooth

blood pressure (4) heart rate and rhythm and (5) rate of energy expenditure of the body These processes altered by emotions may be concerned with the possible resulting damage to the vasculature at sites of predilection One could also evaluate the distribution of different types of emotional make up so called profiles in atherosclerosis as compared to normal persons or persons with other diseases

While all of the foregoing endeavors are fruitful and will advance our knowledge they may not get to the crux of the situation—do emotions lead to and perpetuate atherosclerosis and its close ally hypertension This is what is intriguing us at Michael Reese

Before undertaking any major study it would appear to us that a logical first step would be to outline a working hypothesis This we have done with regard to the role of emotions in atherosclerosis The sequence of the hypothesis is as follows

1 The regulation of body functions by hormones and the nervous system offers a continuous way for the emotions to act chronically

2 Different emotions act differently

3 Different personality types respond differently to the same emotion

4 The emotions and personality types can be classed into a few major groups

5 Dysfunction of regulation of body activity engendered by some emotions—not all—on some personality types—not all—acts alone or via an interplay with other factors to cause vascular disease

6 Dysfunction of bodily regulation engendered by emotions acts in a cumulative manner and leaves somatic residues like hypertensive and atherosclerotic vascular diseases

7 Conditioning and deconditioning in the Pavlovian sense play a significant role in the effects of emotion on cardiovascular reactions

8 Recognition of these effects will ultimately lead to correction of the difficulty to alleviation of the disease and to its prevention

So much for the hypothesis The question is how far is it true and how easy is it to test its various aspects experimentally and in other ways This will depend upon the answers to the following questions

1 Can emotional personality types be classed in a few major groups for statistical study in man?

2 Can emotions be so classed in a few major groups?

3 What exactly is the effect of emotions on animals and on man as far as the several biological parameters are concerned?

4 Do different emotions operate differently?

5 What is the effect of conditioning on heart rate blood pressure heart rhythm lipid metabolism blood clotting etc ? What is the effect

diseases it is worth emphasizing that atherosclerotic disease and hypertensive disease are two different processes closely intertwined and interacting in many persons in the United States but nevertheless two etiologically distinct entities Extensive experimental clinical and epidemiologic data support this conclusion Why the two diseases frequently occur in the same person in countries like the United States is an important unsolved problem If we clearly acknowledge the fact that these are two different diseases (a fact not at all clear 20 years ago) then we have a sound foundation from which to build in our attempt to analyze the role of emotions in the pathogenesis and causation of each and in the interrelationships between the two

WERTHESSEN This is a question to Dr Stamler Am I to understand that in your last remark you drew a clean cut distinction between atherosclerosis as defined as a lesion of the blood vessels and a coronary accident or a cerebral accident as sequelae of atherosclerosis and thus secondary diseases?

KATZ I would like to say one thing Atherosclerosis is one disease while hypertension is another disease It is our working hypothesis that as far as atherosclerosis is concerned hypertension is an accelerator As Dr Stamler pointed out in his formal presentation atherosclerosis occurs in animals in hypertension only in the presence of an atherogenic diet and not when the diet is not atherogenic and this is applicable to man In the absence of atherosclerosis its sequelae cannot occur The fundamental disease is hypertension or it is atherosclerosis The sequelae following these diseases lead to clinical manifestations One other thing in the case of emotion it is my firm conviction that any kind of emotional stress without an atherogenic diet in the past will not cause sequelae since atherosclerosis will not be present You dont get the sequelae of coronary disease unless you have coronary disease to begin with

STAMLER One further point on the cerebrovascular disease problem A degree of confusion is being created at present by the failure to recognize an important fact, i e the cerebral vascular vital statistics from different parts of the world are a summation of deaths due to at least three major processes embolism cerebral hemorrhage and cerebral thrombosis (plus such numerically insignificant processes as cerebral hemorrhage due to ruptured congenital aneurysm) It is therefore not correct to regard all cerebrovascular deaths as having atherosclerosis as the underlying pathologic process This is in contrast to the situation with respect to coronary heart disease where it is proper to equate coronary deaths with atherosclerosis since at least 90% of myocardial infarction deaths exhibit atherosclerosis with its complications as the underlying pathologic process That is not so for strokes

Why is this point worth emphasizing? Consider the problem posed by some of the available epidemiologic data Why is it that the Bantus and the Japanese have a low incidence of coronary disease yet a high incidence of cerebrovascular disease? If the simple sequence taught to most of us in medical school is valid i e hypertension leads to intensified atherogenesis with weakening of the arterial wall and eventual hemorrhage—if this is in fact the actual sequence of events in the cerebral circulation then why doesnt the hypertension lead to aggravated coronary atherogenesis with high rates of myocardial infarction in Bantus and Japanese who do have a high incidence of hypertension and stroke but not coronary disease The same enigmatic problem presents itself with respect to middle aged American women who also exhibit a high incidence of hypertension and stroke but a low incidence of myocardial infarction I would like to focus on a few facts that may serve as a basis for elucidating this set of problems and then suggest a hypothesis First

muscle behavior evidences of different systemic diseases in certain individuals su h as migraine headache which is a vascular smooth muscle reaction peptic ulcer ulcerative colitis essential hypertension and asthma all of which are smooth muscle responses in different individuals subjected to the same emotional situation The psychiatrist also speaks of anorexia nervosa and of exogenous obesity as being basically emotional problems We all know that the blood glucose in diabetics and the blood lipids in California accountants also vary with stress with emotional problems Now my question in leading up to this is with the general impression that I have essential hypertension induced by emotional stress over many years is associated with cerebral vascular accidents and hypertensive cardiorenal disease In a large percentage of these people in a Charleston South Carolina community where we have approximately a 50 50 ratio of Negroes to Caucasians we have observed 4 times the death rate from coronary thrombosis in white males as in either colored males or females In contrast to that is seen an almost 5 fold difference in the death rate among the colored over the white from cerebral vascular accidents and hypertensive cardiorenal disease Do you think that perhaps this ethnic difference might be organ specific in reaction to stress? Where in our group the coronaries might be protected and the cerebral and renal vessels injured or vice versa? Would you care to comment on the ethnic discrepancy or the converse situation between these two ethnic groups?

KATZ I would like to say one thing and then make a comment The one thing I would like to say is this I think it should be put in the record that when Dr Watt at the National Heart Institute was in Russia on a tour he visited a laboratory in which monkeys were being studied under frustrating circumstances A male monkey was separated from his harem and his offspring and saw another male introduced in his place In another experiment the day was changed from a 24 hour day to a much shorter one Under both of these circumstances as I understand it persistent hypertension was produced in these monkeys lasting after the emotional frustration was removed and the father and his family were reconciled and the normal 24 hour day was restored This is simply to document that there are ways by which one might study emotional stresses As to the other question I will turn this over to Dr Stamler

STAMLER A thorough discussion of the very interesting problems posed by Dr Boyle would perhaps take us rather far afield I will restrain myself and make only one or two observations First it should be noted that there seem to be certain differ nces in the epidemiologic patterns of coronary heart disease in Negroes and whites in Chicago and Charleston I say seem to be because the definitive facts are not yet available to us From the mortality data arteriosclerotic heart disease is at least as common in middle aged Negro men as in white in Chicago and more common in middle aged Negro women than white May I call your attention to the graphs presented by Dr Ruth Pick which document this point If one attempts to arrive at a judgment on this epidemiologic problem from a review of the literature one comes away quite frustrated The many available papers are about equally divided as to whether coronary disease occurs less frequently or as frequently in Negroes compared to whites Aside from shortcomings in methodology apparent in many of these reports the disparate findings might well be reflecting actual differences in different parts of the country at different times and under different socioeconomic conditions It is hoped that multiple cooperative efforts in the years ahead will clarify this important epidemiologic problem

Second in any discussion of the role of emotions in the etiology of cardiovascular

disease if it were not for thrombosis I have been slightly surprised to hear nothing much about the effects of endocrines on thrombosis I would suggest that, if any extensive observations are going to be made which will consume years and years a great deal of attention should be paid to the mechanism by which we die that is to say thrombosis My recollection of physiology is pretty dim now but I think it was Cannon 30 40 50 years ago who made the statement and produced the evidence that if animals got frightened their blood clotted more quickly Now that obviously is an endocrine effect I would plead for a few simple observations to be made on thrombosis and the clotting mechanism and if the clotting mechanism is going to be investigated to get first-class hematologists to do it I associate with some first class hematologists but I don t understand what they are saying half the time Most of the clotting tests which are now being used are of no use You can do a great deal better one test, for example the thrombin generation test, is rarely used but it apparently gives one a great deal more information about what is happening to blood than does breaking tubes and seeing when the blood is clotted

KATZ Thank you very much, Sir Howard In my notes I have included hematologists among the people we want in this interdisciplinary cooperative study I am sorry I did not mention them We have added a hematologist to the Chicago Heart Association Committee on Emotions and we are cooperating at the Michael Reese Hospital with our hematologists

with respect to the data on American men and women although there is no sex differential in death rates for cerebrovascular disease over all there is a differential in rates for cerebral thrombosis As with coronary thrombosis cerebral thrombosis—a process having atherosclerosis as its underlying pathological basis—is more frequent in men than women Second the recent pathologic work of Dr Raymond Adams in Boston has yielded findings indicating that in hypertensive cerebral hemorrhage the pathology is not vessel rupture secondary to atherosclerosis Rather it is rupture at the site of a miliary aneurysmal lesion apparently unique to hypertension If this is so then it may be hypothesized that in hypertensive Bantu Japanese and American women a high incidence of cerebrovascular disease is recorded because of a frequent occurrence of this miliary aneurysmal lesion of hypertension leading to cerebral hemorrhage irrespective of the presence or absence of significant cerebral atherosclerosis Thus the epidemiologic data would be accounted for I don t know whether this explanation this hypothesis is valid or not It needs to be tested by further pathologic work in places like the United States Japan South Africa I would very much like to hear the comments of the pathologists on this problem

HOLMAN I will make these comments very brief because this discussion could go on for years I believe that your basic concept of cerebral vascular accident is correct that there are differences between atherosclerosis hypertension aneurysms and so forth The potential common denominator in this discussion is the metabolism of the smooth muscle cells I think that this constitutes a large area for fruitful experimental studies For example we do not know how a single smooth muscle cell arises and inserts how smooth muscle cells perform work how they maintain increased tone how they lead on to hypertension that could lead on to an aggravation of atherosclerosis

We are all fundamentally concerned with these mesenchymal cells in the inner layers of the arterial wall I purposely said "mesenchymal cells because I think the distinction between fibroblasts phagocytes and smooth muscle cells has been overemphasized I am perfectly willing to believe that any one of them can become another and vice versa Thus I would like to suggest to you—and I am certain that you have already thought of it—that you stimulate fundamental studies on smooth muscle

KATZ I agree with Dr Holman—smooth muscle is an important subject for research a most neglected one If some of the effort and money spent on the study of the heart were diverted to a study of smooth muscle of the blood vessels we would be further ahead This was recognized some years ago by the Council on High Blood Pressure Research of the American Heart Association which set up a 2 day meeting on the subject of smooth muscle a few years ago At this meeting the need of further study was stressed Dr Holman I think that was a wonderful statement you made

MARMORSTON As I understand Dr Katz discussion it is mainly centered around emotions in relation to these diseases and therefore there are the normal emotions or the normal emotional reactions to situations and the excessive overtones of emotions I wonder how much attention has been directed by cardiologists to this phase of the problem in their treatment of patients with coronary artery disease I mean specifically psychotherapy How much of this kind of work has been done and is being done by scientists and investigators in this field?

FLOREY This seems to be the session in which all sorts of irrelevant matters are being thrown into the hat Perhaps I might be able to throw in one now I suppose that a case could be made out that atherosclerosis would not be a very serious

CHAPTER 28

Some Aspects of the Endocrinological Picture of the South African Bantu — A Population Relatively Free from Mortality from Coronary Disease

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INTRODUCTION

In this paper it is intended to begin by providing a background of general information on the local Bantu so that afterward the aspects of their endocrinological picture to be described will be better appreciated I am doing this first because the Bantu comprise an excellent base line for etiological research on account of their relative freedom from death from coronary heart disease Secondly while some of the research contributions have been published overseas others have appeared in less accessible local journals moreover some of my information concerns studies still in progress In short I wish to give a very brief account of investigations made by various research groups on the Johannesburg Bantu in respect to their mortality from coronary disease the incidence of atherosclerosis some aspects of their biochemical picture their diet and possibly other influencing factors Against this background I will then give specific information on their liver disease picture estrogen and 17 ketosteroid excretion patterns and the incidence of gynecomastia and then seek to discuss the significance of these aspects in regard to the freedom of these people from death from coronary thrombosis

The Bantu of South Africa number about nine million thereby greatly outnumbering the white population of about three million In addition there are about a million Eurafricans and a half million Asiatics Of the Bantu roughly a third dwell in Native Reserve areas about a third work on farms owned by white people and the remainder are urbanized working in government and municipal departments in industry as domestic servants etc These people therefore may be observed in various stages of transition of diet and manner of life from the primitive living off the land to the most sophisticated of urban houseboys

MORTALITY FROM CORONARY HEART DISEASE

In 1946 in Johannesburg Becker (7) noted only 1 death from the disease among 362 necropsies on nonwhites (predominantly Bantu) over 50 years of age More recently from the same city Higginson and Pep

(33) in a more detailed study reported severe atherosclerosis of aorta and coronary system to be much less common in Bantu compared with American and Danish hospitalized populations Thus they found occlusion of the coronary arteries in 1 6% of patients at 40 to 59 years and 3 0% in patients 60+ years compared with 12 8 and 13 3% respectively found in 3400 necropsies of hospitalized American white subjects studied by Gordon *et al* (29) at Massachusetts General Hospital Further in the study of Wanscher and co workers (58) on Danish hospitalized patients over 60 years severe atherosclerosis of aortas and coronaries was present in 58% males and 66 5% females corresponding Bantu figures were 19 and 6% respectively (33) More recently Anderson *et al* (1) have investigated the age trend of changes chemical and pathological in comparable series of aortas from Bantu and white patients 10% of Bantu against 47% of white subjects showed aortic "ulceration with calcification and or widespread involvement of entire surface"

Regarding chemical composition the workers (1) just cited have found that in groups of aortas graded equally (naked eye assessment) under conditions where *mild to moderate* atherosclerosis prevailed mean percentage composition was similar for both races Yet in *severe* atherosclerosis with similar surface involvement percentage composition in the races differed considerably increases in dry weight ash calcium and cholesterol being much more marked in the white series Since all these changes in the Bantu (sometimes very marked) occur within a context permitting only a very low incidence of fatal coronary episodes it may be assumed that in this particular restricted sense lesions present betoken aging and degenerative changes of little significance to health. In this connection it is interesting to note that Pepler (46) found medial degeneration in the aorta of Bantu to be present in *all* subjects over 10 years the inner third being most frequently affected

Regarding the sex bias of atherosclerotic lesions Becker (7) reported no male predominance In the study of Higginson and Pepler (33) severe aortic atherosclerosis with or without marked atherosclerosis of the coronary arteries was found to be more common in females than in males although the most severely affected of arteries were those of males

It must be strongly emphasized that the disparity in atherosclerotic lesions between Bantu and white subjects is small compared with the very wide disparity in death rates from coronary disease

Before leaving the subject it must be made clear that *cerebral* atherosclerosis in the Bantu is common and occasionally severe it is responsible for many deaths although the precise mortality rate is not

ler (33) reported that among 1328 necropsies of Bantu adults dying in a large local nonwhite hospital (1500 beds) there were 4 deaths from coronary thrombosis among 807 males and 4 deaths among 521 females In comparison at Minneapolis among 22 149 postmortems of hospitalized patients carried out by Clawsen and Bell (21) the death rate from the disease during the period 50 to 69 years was 23 times higher in males and 7 times higher in females than in corresponding Bantu groups Among the *nonhospital* Bantu population current local information (2) indicates a coronary disease death rate of 6 per 100 000 (not all verified by necropsy) In strong contrast among the total white population in South Africa the present death rate from the disease is about 142 per 100 000 (50) the figure for the total population of the United States is 228 per 100 000 (45) Of course the more youthful character of the Bantu must be taken into account However it must be pointed out that the occasionally expressed view that the Bantu do not die from coronary disease merely because they do not live long enough is wholly incorrect In Johannesburg for example with over half a million Bantu there are approximately 80 000 between 45 to 60 years and 15 000 over 60 years (53) Comprehensive electrocardiograph studies undertaken in Cape Town (48) confirm the rarity of myocardial infarction in the Bantu

The above figures concern *urban* Bantu Among rural Bantu the disease appears to be even less common Thus in many country mission hospitals coronary thrombosis in a Bantu has not yet been encountered In territories north of South Africa e g Uganda in 1956 Trowell and Singh (52) reported no proven case among 6500 necropsies

There appears to be no obvious sex bias of deaths from the disease among urban Bantu (33 55) although the number thus dying is too small to draw significant conclusions Yet observations by Keys (36) do suggest that in populations (at least in certain age groups) with a relatively low compared with a high death rate from the disease the sex ratio tends to become narrower

Briefly then the South African Bantu are a population among whom coronary heart disease mortality presents no serious public health problem

The Incidence of Atherosclerosis

Among these people atherosclerosis certainly *does* occur occasionally severely In 1946 from an examination of 3000 consecutive necropsies of nonwhites (predominantly Bantu) ranging from birth to 60+ years Becker (7) found atherosclerotic lesions in 27 6% lesions were the direct cause of death in 0 4% Later Higginson and Pepler

various vegetables (pumpkin, sweet potatoes (*Ipomoea batatas*) and greens). The amounts of eggs, meat, fish and dairy produce eaten, also sugar, usually are much less than among the white population. Briefly speaking, very generally the diet of these people according to accepted standards, although usually adequate in calories and gross protein, is low in animal protein and fat (and cholesterol), high in carbohydrate and crude fiber, high in certain mineral salts and vitamins, e.g., phosphorus, iron, vitamin A and thiamine, but low in others, e.g., calcium, riboflavin and vitamin D. The fat moiety, roughly 25 to 60 g. per diem, supplies 15 to 20% of calories and is derived largely from vegetable sources. It should be understood that this pattern of diet is common; in fact, qualitatively it is the pattern consumed by the greater part of the world's population. The same pattern of diet was consumed by our forefathers a century or so ago; moreover, the adoption of such a regimen frequently is forced upon many countries for limited periods by wartime restrictions.

Influencing Factors Other Than Diet

Some of the possible influencing factors, other than diet, will now be mentioned very briefly.

Hypertension

Becker (6) found hypertensive arteriosclerosis to be common, occurring in 8.2% of a series of 3000 routine necropsies on nonwhite (predominantly Bantu) subjects. Hypertensive heart disease was the most common cardiovascular disease, being responsible for one third of cases of congestive failure and accounting for 6% of deaths in his series. Becker stressed that "in spite of the fact that the coronary vessels are frequently diseased in condition and the myocardium as frequently gravely damaged" coronary thrombosis was still very rare.

Coronary Vessels

Brink (17) has produced evidence in adult Bantu of a third primary division of the left coronary artery present in three quarters of a small group of hearts examined by the Schlesinger technique. Singer (49) in Cape Town, however, has found the same coronary branch anomalies in white hearts, and local opinion tends to the view that the presence of the abnormality is of little etiological importance in the subject under discussion.

Smoking

Smoking is common among the Bantu. Incidentally, Higginson and Oettle (32) have noted cancer of the lung to be about half as common among Bantu compared with the population of the United States

known There can be no doubt that the factor or factors protecting certain population groups from death from coronary heart disease do not protect correspondingly from death from cerebral thrombosis and hemorrhage Thus death rate from the latter causes in France is much the same as that in England and Wales Sweden and Denmark yet death rate from coronary disease in France is roughly only a quarter of that in the other countries mentioned (45)

The Biochemical Picture

First in regard to serum—whereas observations show that average cholesterol levels are the same in newborn babies of both races levels differ significantly at 30 to 40 years when Bantu values reach a plateau yet those for white subjects continue to show an upward trend (12 57) The same pattern of changes has been found by Bersohn and co workers (9) *to characterize serum phospholipid cholesterol phospholipid ratio* and to a lesser extent beta lipoprotein concentration indicated by ultra centrifuge flotation rates (35) A recent study by Antonis (3) on the relative concentrations of polyunsaturated fatty acids in sera and plasma from Bantu and white subjects has revealed the former to have higher levels of dienoic and tetraenoic acids (linoleic and arachidonic acids) An important recent observation by Gillman and co workers (28) is that plasma fibrinolysin activity is unusually high in the Bantu

In urines Bersohn and Oelofse (10) have reported a significantly higher excretion of total estrogens per 24 hours in young urban Bantu males compared with young white subjects but this aspect will be en larged upon later

Regarding stools the indigenous diet of the Bantu is one of 'high residue' yielding with rapid passage voluminous stools (54) contain ing larger amounts of total fat (54) and according to Antonis and Bersohn (4) larger amounts of sterols bile acids and total fatty acids compared with stools from white subjects However there is evidence that the same type of diet evokes similar response in white people (4 26 42 54)

The above and other aspects of the general biochemical picture that could be described may be regarded as eminently favorable" in the sense that they are in consonance with a very low death rate from coronary disease

The Diet of the South African Bantu

The Bantu diet includes a large amount of cereals supplying 50 to 90% of the calories Such cereals usually whole ground or lightly milled comprise maize (corn) wheat and 'kaffir corn" (*Sorghum vulgare*) Other items of diet include different types of legumes and

hepatic conditions namely portal fibrosis and siderosis (abnormal iron deposition) are present in the majority of adult Bantu males and in a somewhat smaller proportion of adult females (31)

Biochemically many workers have noted that the serum protein picture is characterized by relatively low albumin and high globulin (particularly gamma globulin) fractions such being present in the majority of Bantu although the picture at birth is normal (13) This abnormality apparently prevailing throughout life would seem to imply liver dysfunction in this particular respect

Excretion of Urinary Estrogens

It is believed that one of the many functions of the liver is to inactivate hormones produced by the adrenals and gonads (20) In view of what has been indicated already naturally much interest attaches to estrogen *excretion levels in the Bantu* This subject *is being extensively* investigated at this Institute by Bersohn and Oelofse (10) Their findings on 21 local urbanized outwardly healthy Bantu males compared with an equal number of white subjects are summarized in Table I

TABLE I

Excretion of Estrogens Per 24 Hours by Young Urban Bantu and White Subjects

	Bantu subjects Mean standard deviation % of total estrogens and range	White subjects Mean standard deviation % of total estrogens and range
Total estrogens	11 5 ± 4 2	8 0 ± 3 2
(µg)	(5 0–21 0)	(3 8–15 4)
Estradiol	2 5 ± 1 8 (21 8%)	1 1 ± 0 9 (13 9%)
(µg)	(0–6 4)	(0–3 1)
Estrone	5 5 ± 2 6 (47 8%)	4 3 ± 2 1 (53 7%)
(µg)	(2 0–11 3)	(1 7–9 0)
Estriol	3 5 ± 1 4 (30 4%)	2 6 ± 2 1 (32 4%)
(µg)	(0 8–6 4)	(0 4–9 9)
Number of subjects	21	21
Age	31	31
(Years)	(21–45)	(20–48)

Both total estrogens and of the fractions estradiol are significantly higher although not spectacularly so in Bantu as compared with white subjects Many of the same observations have been made by Bloomberg and co workers (15) they have noted somewhat more elevated levels in the Bantu

Stress

The Bantu have plenty of stresses some similar to our own but others peculiar to themselves related to witchcraft taboos etc

Physical Activity

It is generally agreed that the level of physical exercise is an influencing factor in the disease under discussion Although the Bantu pursue a more active life than do white South Africans their motor fitness (e g performance on Harvard Step Test etc) *far exceeds* that of the white population (39 41 44) The explanation is not clear However *it is surely of relevance that muscular exercise increases fibrinolysin* activity (30 43) the high level of the latter in the Bantu has been mentioned already (28) Furthermore the recent study of Connell *et al* (22) indicating a depression of 17 ketosteroids in men habituated to physical activity may be apposite in the Bantu context and will be referred to later

The prime differences between the Bantu and white populations therefore would seem to concern their diet and physical activity

Endocrinological Aspects

In the previous section in considering factors other than diet it would have been appropriate to have discussed the role of liver disease for it is widely believed and recently has been restated (27 28) that apart from Bantu diet and activity liver disease and liver abnormality in these people with their ramifications in "over estrogenization" and low 17 ketosteroids and in the commonness of gynecomastia and other phenomena have a strong bearing on the infrequency of severe atherosclerosis *and rarity of coronary disease among them* The obvious question that arises is—how significant is the part played by liver disease and abnormality in "protecting" these people? Are the hepatic changes and their ramifications of primary protective importance? Or on the other hand do they merely confer additional protection within a context in which diet and activity are the principal protecting factors? Or are the two aspects inextricably bound up together? Ones endeavor to throw light on this problem forms the substance of this contribution

Liver Disease and Abnormality

Among Johannesburg Bantu the incidence of primary carcinoma of the liver is 14 4 per 100 000 (32) and accounts for about 1 5% of deaths Among *hospitalized* patients cirrhosis occurs in a mean of 9% of males over 10 years and 5% of females over 40 years (31) Two *symptomless*

Data on serum protein fractions and certain precipitation tests have revealed no greater abnormality in subjects affected with gynecomastia than in subjects not affected In Bantu subjects with slight or moderate gynecomastia a few determinations of total estrogen and 17 ketosteroid excretion by Bloomberg and co workers (16) have revealed values not significantly different from that of Bantu without gynecomastia In other words the biochemical picture thus far investigated appears to be nonspecific

There is no doubt that the type of gynecomastia seen in the Bantu of Southern Africa differs in several respects from the condition sometimes noted in (*a*) chronic liver disease (20) (*b*) rehabilitation from malnutrition and undernutrition (38) and (*c*) estrogen therapy The common view that gynecomastia in the African is an expression of habitual protein malnutrition (18) cannot be accepted as established Likewise the view that hepatic disease in these people impairs the normal inactivation of hormones thereby leading to increased excretion of estrogens and hence promoting the occurrence of gynecomastia requires much further work before acceptance Hepatic disease and abnormality are common enough in nonwhite people in other parts of the world but gynecomastia as seen in the African Bantu apparently is not reported Possibly injury to the liver and to other organs sustained by Bantu infants at weaning time and thereafter may provide a substrate favorable for the development of gynecomastia in later life But the nature of the presumably local precipitating factors is not known although Becker (8) at this Institute has suggested that the estrogenic and other properties of the numerous herbal preparations used by these people merit serious investigation

COMMENT

The general context including the factors Bantu diet and activity low serum lipids high plasma fibrinolytic activity a high incidence of liver disease and abnormality and "feminization (elevated estrogen and low 17 ketosteroid excretions gynecomastia atrophy of the testes) at first sight would seem to form the basis of an attractive hypothesis possibly to account for the rarity of coronary occlusion in these people (27 28) As noted earlier the problem requiring elucidation is—of the protection conferred by the above context how much is due to diet and manner of life and how much is contributed by superimposed liver disease and abnormality with their varied ramifications? In seeking to answer or at least to throw light on this problem the following considerations seem relevant

Excretion of 17 Ketosteroids

17 Ketosteroids values appear to be slightly lower in Bantu compared with white subjects On 20 Pretoria urban Bantu males (mean age 26 years) Kinnear (37) found a mean of 11 8 mg per 24 hours which may be compared with a mean of 15 mg reported in an American study cited by Cantarow and Trumper (20) Bloomberg and co workers (15) have also found low values in urban Bantu values being lowest in patients with cirrhosis An incomplete local study by Politzer and Louw (47) however undertaken on probably more sophisticated Bantu indicates no significant difference in mean values for both races Low values have been reported for groups of West Africans (5) and also Indians (25) The possibility that the greater motor fitness of the Bantu contributes to depress their 17 ketosteroids levels cannot be excluded

Gynecomastia

The picture of liver disease and abnormality and of excessive estrogen excretion and possibly decreased 17 ketosteroid excretion, now leads to the subject of gynecomastia (enlargement of the male breast) a phenomenon common among the Bantu Locally Higginson and Simpson (34) have noted a discrete mass 2 cm in depth to be present in 2% and some abnormality present in 33% of necropsies of Bantu adult males dying in hospital Exceptionally the condition occurs in a very severe form (i e breasts being 10 to 15 cm in diameter)

With collaborators I have studied 21 subjects all young men (56) They appeared in good nutritional condition with no evidence of previous dietary privation Enlargement generally developing in late puberty usually was bilateral nontender with no exudate Seven subjects maintained that the condition was present in their fathers and occasionally in their brothers thus introducing a possible familial factor into the etiology Almost all subjects asserted that they had libido and 7 maintained that they were fathers of children Hair in axillae and pubes appeared masculine in distribution Apart from enlarged breasts body configuration was likewise masculine In all cases save one the penis appeared normal Slight atrophy of testes appeared present in a few subjects In all subjects the voice had broken Seven subjects had slight enlargement of the liver but this is not believed to have etiological significance due to equal commonness of hepatomegaly in Africans without gynecomastia Higginson and Simpson (34) have noted no correlation between gynecomastia and liver disease Incidentally Higginson and Oettle (32) have found carcinoma of the male breast to be no more common among Bantu than in white males

More apposite Bloomberg *et al* (15) found no significant difference in Bantu with and without cirrhosis (*b*) The superior motor fitness of the Bantu cannot be excluded from playing some part in the levels ob served if as has been shown on white subjects a habitually high level of physical activity can depress 17 ketosteroids values very significantly (22) it would seem reasonable to consider that other aspects of the endocrinological picture likewise may be affected (*c*) Next there is the question of the influence of pattern of diet on metabolism Among the Bantu we have encountered many abnormalities" almost certainly caused by the type of diet consumed moreover there is limited al though increasing evidence that white subjects habituated to the same type of diet also can become marked by these abnormalities It would therefore evoke little surprise to find that the type of diet consumed by these people is partly or even largely responsible for the bio chemical endocrinological changes under discussion and studies on these aspects are already in progress (11) Briefly then *in seeking to apportion responsibility for the endocrinological changes in the Bantu caution is required before attaching too much blame to liver disease and abnormality*

5 It must be repeated that despite the commonness of liver disease and abnormality in Middle and Far East populations *gynecomastia* as seen in the Bantu apparently is not observed This abnormality would seem therefore to be a local phenomenon the etiology and significance of which are still obscure

6 As already indicated evidence suggests that white subjects once they become habituated to a Bantu type of diet take on many of the metabolic features characteristic of these people Such an adaptation ap parently is not without its reward In Pretoria Gaol a diet of similar pattern is consumed by both Bantu and white prisoners A collabora tive study under the leadership of Prof P J Kloppers has shown a closely similar biochemical picture (4 9 11) at that prison (the largest in the country) no long term white prisoner has yet died from coronary thrombosis

Briefly then it is suggested that the Bantu are protected against severe atherosclerosis and coronary disease mortality primarily by reason of their diet and manner of life and that the protection conferred by liver disease and abnormality is supplementary and of much less sig nificance

Summary

The South African Bantu are a population almost free from mortality from coronary heart disease In order to provide a background con cerning these people brief information has been given on the incidence

1 In the first place, it is contended that different populations suffering little from coronary heart disease mortality need not be protected by the selfsame factors and that different contexts of diet and manner of life may well yield the same end result Thus whatever explanation accounts for the favorable position of the Bantu need not necessarily obtain with other equally favored populations

2 Among our forefathers, who consumed a diet similar in many respects to that of the Bantu *angina and coronary thrombosis were not the problems that they are today* Moreover, in certain Scandinavian countries wartime restrictions imposed consumption of a diet also similar *in many ways to that of the Bantu such changes being accompanied by* a lesser degree of severe atherosclerosis and reduction in deaths from coronary disease (14) *It is highly improbable that protection with either our forefathers or the Scandinavians was conferred or appreciably augmented by a high incidence of liver disease and abnormality*

3 That subjects with *cirrhosis* suffer less from severe atherosclerosis and coronary disease has been noted often in the literature (23) A present day western country possibly affected in this manner is France where information suggests (*a*) that cirrhosis is as common or more common than among the Bantu (51) (*b*) that very severe atherosclerosis is not common (51) and (*c*) that the death rate from coronary disease is only 38 per 100 000 i e about one sixth of that in the United States (45) *It would therefore seem reasonable to consider that in countries such as the present day France and in various underprivileged populations in Africa, the Midlde and Far East and elsewhere where liver disease is common that it does contribute a quota of protection to part of the adult population* Part of the protection may well be related to the elevated fibrinolytic activity reported to occur in cirrhotics (40)

4 Turning now to the aspect of *elevated estrogen and low 17 ketosteroids levels*—such may well be due to the interaction of a number of influencing factors (*a*) Regarding the role of liver disease—while as already noted only a small proportion of Bantu suffer from cirrhosis the major proportion have portal fibrosis or siderosis or show some biochemical abnormality referable to hepatic impairment At first sight therefore it is tempting to ascribe much of the responsibility for changes in the Bantu to liver disease and abnormality However although there is ample information that patients with cirrhosis have low levels of 17 ketosteroid excretion the same certainly does not apply to elevated levels of total estrogens (24) In the recent study of Cameron (19) for example total estrogens were found to be within normal limits in 10 out of 12 patients suffering from chronic liver disease

26 Gardner J A and Fox F W Proc Roy Soc B 92 358 (1921)
27 Gillman T Lancet ii 1117 (1957)
28 Gillman T Naidoo S S and Hathorn M Lancet ii 696 (1957)
29 Gordon W H Bland E F and White P D Am Heart J 17 10 (1939)
30 Greig H B W Lancet ii 16 (1956)
31 Higginson J Grobbelaar B G and Walker A R P Am J Pathol 33 29 (1957)
32 Higginson J and Oettle A G personal communication (1958)
33 Higginson J and Pepler W J J Clin Invest 33 1366 (1954)
34 Higginson J and Simpson J W personal communication (1958)
35 Joubert F van Bergen A Bersohn I and Walker A R P (unpublished work)
36 Keys A Symposium on Arteriosclerosis Minnesota Heart Association and the University of Minnesota p 28 (1955)
37 Kinnear A A S African J Lab and Clin Med 2 263 (1956)
38 Klatskin G Salter W T and Humm F D Am J Med Sci 213 19 (1947)
39 Kloppers P J personal communication (1958)
40 Kwaan H C and MacFadzean A J S Lancet i 132 (1956)
41 LeRiche H Riordan D Smit R J Ockerse T Best F Kinnear A A and Walker A R P S African Med J 27 103 (1953)
42 McCance R A and Walsham C M Brit J Nutrition 2 26 (1948)
43 MacFarlane R G and Biggs R Blood 3 1167 (1948)
44 Morrison J F and Strydom N B S African J Med Sci (in press)
45 Mortality due to arteriosclerosis Epidemiol Vital Statist Rept 9 537 (1956)
46 Pepler W J S African J Lab and Clin Med 1 222 (1955)
47 Politzer W L and Louw B Lancet (in press)
48 Shrire V S African Med J 32 177 (1958)
49 Singer R quoted by Bronte Stewart B Keys A Brock J F Moodie A D Keys M H and Antonis A Lancet ii 1103 (1955)
50 Smit Z M S African J Lab and Clin Med 3 29 (1957)
51 Tremoliere J personal communication (1957)
52 Trowell H C and Singh S A E African Med J 33 391 (1956)
53 Union Department of Census and Statistics Gov t Printer Pretoria 1954
54 Walker A R P Nature 164 825 (1949)
55 Walker A R P Andersson M and Bersohn I Brit Med J 1 1234 (1956)
56 Walker A R P Ann N Y Acad Sci 69 989 (1958)
57 Walker A R P and Arvidsson U B J Clin Invest 33 1358 (1954)
58 Wanscher O Clemmesen J and Nielsen A Brit J Cancer 5 172 (1951)

Discussion

STAMLER It is a generally accepted truism that the international outlook and community of science are one of its cardinal strengths After this presentation I know you will agree that the Planning Committee was wise to make this an inter national conference and to invite guests from as far away as South Africa Dr Walker s fascinating paper is now open for discussion

WHITE I want to thank Dr Walker for a most illuminating presentation You indicated I believe at one point that the herbs which the Bantu are prone to con sume may have contained estrogens and I wonder whether you have actually done analyses or assays of the estrogen content of the diet since it is of a vegetable nature

WALKER It is hoped shortly to initiate determinations on the estrogen content of some of their herbal preparations I want to stress that the use of these prepara

of coronary thrombosis on atherosclerosis the biochemical picture the diet and other possible influencing factors Against this background the endocrinological aspects described concern liver disease and abnormality the excretion of estrogens and 17 ketosteroids and the phenomenon of gynecomastia Discussion suggests that the relative freedom of the Bantu from coronary occlusion is due primarily to their diet and activity and not to liver disease and abnormality although the latter may confer additional protection to a small proportion of the adult population Evidence suggests moreover that adoption by white subjects of a diet similar to that of the Bantu imposes an altered metabolic picture which *inter alia* appears to be associated with a reduced death rate from coronary heart disease

References

1 Andersson M Walker A R P Higginson J and Lutz W *Am J Pathol* (in press)
2 Annual Report of Public Health Department Johannesburg City Council 1956
3 Antonis A Repts of 4th Intern Conf on Biochemical Problems of Lipids Oxford Univ Press London and New York July 1957 (in press)
4 Antonis A and Bersohn I personal communication (1958)
5 Barnicot N A and Wolffson D *Lancet* i 893 (1952)
6 Becker B J P *S African J Med Sci* 11 1 (1946)
7 Becker B J P *S African J Med Sci* 11 97 (1946)
8 Becker B J P personal communication (1958)
9 Bersohn I personal communication (1958)
10 *Bersohn I and Oelofse P J S African Med J 31 1172 (1957)*
11 Bersohn I and Oelofse P J personal communication (1958)
12 Bersohn I and Wayburne S *Am J Clin Nutrition* 4 117 (1956)
13 Bersohn I Wayburne S Hirsch H and Sussman C *S African J Clin Sci* 5 35 (1954)
14 Biorck G *in* Cardiovascular Epidemiology p 8 Hoeber Harper New York, 1956
15 *Bloomberg B M Miller K Keeley K J and Higginson J J Endocrinol* 17 182 (1958)
16 Bloomberg B M Miller K Keeley K J and Higginson J *S African J Med Sci* (in press)
17 Brink A J *Clin Proc (Cape Town)* 8 137 (1949)
18 Brock J F *Nutrition Revs* 13 1 (1955)
19 Cameron C B *J Endocrinol* 15 199 (1957)
20 Cantarow A and Trumper M *in* Clinical Biochemistry" 4th ed p 541 Saunders Philadelphia 1950
21 Clawson B J and Bell E T *A M A Arch Pathol* 48 105 (1949)
22 Connell A M Cooper J and Redfearn J W *Acta Endocrinol* 27 179 (1958)
23 Creed D L Baird W F and Fisher E R *Am J Med Sci* 230 385 (1955)
24 Editorial Hormones and liver disease *Brit Med J 1 270 (1958)*
25 Friedmann H C *Lancet* ii 262 (1954)

WALKER First Dr Holman regarding breaking down the word severity" of lesions—we have used the classification of lesions suggested by the Danish workers Wanscher *et al* Next concerning the lesions you have noted in the aorta from young Bantu children—there is no doubt from what I have stated already that atherosclerosis occasionally severe (ulceration and calcification and/or widespread involvement of entire surface) is by no means uncommon among the Bantu Yet with rare exceptions patients with severe lesions have not died from coronary thrombosis You then mentioned about clotting mechanism and thrombosis Dr Greig (at the Institute where I work) in a recent paper in the *Lancet* has referred to the lack of correlation between elevated blood lipid levels and atherosclerosis and considers that the former bear on the etiology of thrombosis rather than on the extent of atherosclerosis In this respect Dr Greig has produced evidence that beta lipoprotein inhibits fibrinolysis the degree of inhibition depending on the composition of the beta lipoprotein which in turn depends on the type of fat ingested In other words if the beta lipoprotein position is favorable and continues to remain so as presumably occurs in the case of the white prisoners described then serious thrombotic episodes are prevented for as stated these life-sentence people do not die from coronary disease In other words if prisoners in altering their diet etc render more active their fibrinolysin mechanism they can well ward off death from coronary occlusion despite occasionally severe atherosclerosis probably being present before entering prison

FURMAN Possibly you have already answered this question In view of the evidence of estrogenism in the Bantu do you find the lipoprotein distribution characteristic of estrogen effect namely most of the circulating cholesterol and phospholipid in the high density or alpha lipoprotein fraction?

WALKER First in the case of the Bantu and white prisoners both of whom as you will remember have been habitually consuming a Bantu type of diet for some considerable time approximate values for both groups found by Dr Bersohn are as follows Of the serum total lipoprotein about one third is alpha and two-thirds are beta lipoprotein Of the cholesterol about one-quarter is attached to alpha lipoprotein and the remainder to beta lipoprotein With phospholipid 35 to 40% is attached to alpha lipoprotein and 60 to 65% is beta lipoprotein phospholipid Limited evidence suggests that the rural Bantu picture conforms to that of these prisoner groups but with urban Bantu accustomed to a varying measure of dietary sophistication there is a trend to approach the picture common to the white population i e higher levels of cholesterol bearing beta lipoprotein In the Bantu and white prisoners the cholesterol phospholipid ratios were 0 80 and 0 84 respectively Briefly then the blood lipid picture of the Bantu is not the same as that associated with the administration of estrogen

STRISOWER There are many very interesting findings in this paper On one of your points mentioned very briefly I would like a little elaboration I am referring to the changes in the serum proteins It was stated that the gamma globulins appeared to be elevated albumin is decreased but this was unrelated to diet and it occurred in preschool children I wonder if you have any explanation for these observations such as perhaps the prevalence of some chronic parasitic infestations or any other chronic disease which we know increases the gamma globulin fraction Also could you elaborate a little more on the age distribution and if you have the data on the distribution of the other electrophoretic globulins?

WALKER The serum protein picture for both black and white babies is the same at birth From the period of about 1 year of age until 2 to 3 years when the nutritional situation of children is at its worst throughout their life span it is a

tions is widespread although of course differing from region to region Our Bantu mineworkers come from different African territories and some individuals bring veritable portmanteaus of herbal preparations the source of which they are often ignorant or won t divulge In South Africa we fully realize the importance of knowing more of the composition and pharmacological action of these preparations The estrogen content of the diet of the Bantu has not yet been investigated

WHITE May I ask one more question Have you the opportunity to do liver function tests?

WALKER Yes whole batteries of liver function tests have been carried out and there is no significant difference between Bantu with and without gynecomastia

HOWARD To supplement Dr White s question I presume that the prisoners who were on the Bantu type diet did not have the opportunity to have any unusual herbs so that it would seem logical that the diet was a more important factor than herbs Is that correct Dr Walker?

WALKER *Yes that is correct I believe that the type of diet consumed is the* important factor The prisoners did not have access to medicinal herbs I must also make it clear that significant gynecomastia is not a feature of the prisoners certainly not more so than nonprisoner Bantu of the same age group

BOYLE Would you please give some estimation of the physical activity of these prisoners Are they confined or are they laboring prisoners?

WALKER The white prisoners pursue manual craftwork woodwork cobbling etc The Bantu do possibly more active work gardening, shifting soil and the like but they certainly don t overwork On these black and white prisoner groups certain motor fitness tests have been carried out the Bantu seem virtually in exhaustible for example when carrying out the Harvard Step Test, in comparison to the white prisoners Yet the diets of both groups have much the same composition and there is not a gross difference in habitual activity between the groups

HOLMAN First I would like to compliment Dr Walker on his excellent presentation of a very difficult subject I would also like to put in a plea that you break down the expression severity of lesions into different categories or stages We have found from the studies in Guatemala (in collaboration with Dr Carlos Tejada) for example that the children with kwashiorkor may have striking fatty streaks The presence of malnutrition does not preclude the presence of fatty streaks but this does not mean necessarily that these fatty streaks will progress to clinical disease If we are going to dissect out the factors involved in atherosclerosis and ultimately correlate them with the natural history of the disease I think that each step in the process—(*1*) fatty streak (*2*) fibrous plaque (*3*) complications of lesions and (*4*) clinical disease—is going to have to be recognized

To come back to the question that Sir Howard raised namely thrombosis maybe this is the mechanism of conversion of fatty streaks to fibrous plaques and certainly thrombosis is one of the major complications The factors governing thrombosis may have little or nothing to do with the formation of fatty streaks In the anatomic material which we have received thus far from Dr Wainwright in Durban South Africa we have found the usual amount of fatty streaks The amount of this material (principally aortas) is not sufficient to treat statistically and this is especially true of the fibrous plaques and later stages But if we accept the morbidity and mortality statistics showing a greatly reduced incidence of myocardial infarction among the Bantu and at the same time accept the data showing the usual amount of fatty streaking in the Bantu we must recognize that factors other than those that initiated the process (atherogenesis) must be involved in the evolution of these lesions to the final stage of clinical disease

WALKER There are a few data published on kidney function in Bantu children with kwashiorkor A detailed study on adults has not yet been undertaken although information on hospitalized Bantu does not indicate widespread impairment

EDER I should like to ask two questions The first is what is the sodium chloride intake of the Bantu diet and the other is did you measure estrogen excretion on the white patients?

WALKER The intake of salt by the Bantu differs enormously from region to region Bantu mineworkers about whom I have spoken before are housed in com pounds In front of each compound kitchen is placed a barrel of salt It has been observed that laborers from some territories take almost handfuls with their meals whereas other groups take little or none After discussions with Dr Stamler it will be our endeavor to study various tribal groups in relation to salt intake hypertension prevalence of cerebral lesions etc Estrogen excretion levels have been carried out on both the white and Bantu prisoner groups Dr Bersohn has found results in both groups to be equally elevated Since the results obtained are so unusually high steps are being taken to check and recheck them I would therefore prefer not to give the actual figures at present

KATZ I would like to ask Dr Walker if he can fractionate the food materials he gives his prisoners to locate the possible estrogenlike material in the food or a possible anticoagulant material Is there something in the grain they ingest which is estrogenlike or anticoagulant?

WALKER The question of the presence of estrogenlike material in the food and also of components which might be considered anticoagulant will certainly be studied once the precise biochemical picture of the prisoners has been established

WERTHESSEN Did I understand you correctly to say that cerebral athero sclerosis is marked in the Bantu? Do you find in autopsy material atherosclerosis in the cerebral vessels with aortae similar to the one you pictured?

WALKER Insufficient work has been done to answer that question satisfactorily It is evident from studies by Drs Laurie and Woods in Natal that cerebral athero-sclerosis in the Bantu can be very marked and responsible for many deaths In Johannesburg on the basis of death certificate data I have calculated that the age specific death rate from cerebral catastrophes during the period 45–64 years actu ally is higher than the corresponding figure in New York mortality from hemorrhage is much greater than that from thrombosis and embolism The classification of lesions in aorta in coronary and in cerebral vessels in the same series of cadavers has not yet been undertaken

WERTHESSEN I think you see what I am seeking namely a genetic difference between two groups I am therefore raising the question Is there a possibility that this race of people happens to respond to grains differently from the way we do?" I don t think you can neglect the important genetic factor here

WALKER That possibility is perpetually before us Nevertheless while I agree that you cannot neglect the genetic factor all our experience with the Bantu sug gests that racial or genetic differences are not of significance in the subject under discussion

ROBINSON Have you observed testicular atrophy in the male Bantu with gyne comastia?

WALKER Yes a study of testicular atrophy and its correlation with gynecomas tia has just been completed by Drs Higginson and Simpson at Johannesburg The two conditions are often although by no means invariably associated together

STAMLER May I on behalf of everyone present thank Dr Walker again for his very valuable presentation

common feature for the albumin to fall and the globulins especially gamma globulin to rise Thereafter the altered picture persists despite adequate feeding and various reports indicate that this reversed albumin globulin ratio prevails among from half to three quarters or more of the adult population Infection during the period mentioned especially gastroenteritis undoubtedly aggravates the position but parasitism is not a factor at least it is not the problem prevailing in Central Africa In young Bantu children with or slightly recovered from kwashiorkor the globulins as percentages of total serum protein are distributed (electrophoretically) approximately as follows alpha 11% beta 13% gamma 26% Among the older population e g the Bantu prisoners Dr Bersohn has found serum globulins as mean percentages of total protein to be as follows alpha 1 7% alpha 2 10% beta 15 5% and gamma 19% Among the white population speaking generally reversal of albumin globulin ratio is infrequent perhaps less than 10% being affected beta and gamma globulin fractions are of course lower

BOYD If Bantu with gynecomastia are contrasted with those who do not have gynecomastia and matched for age and physique do you find any difference in estrogen excretion in these two groups and does this level of estrogen excretion correlate with the serum cholesterol level? And one very minor point since estrogens elevate the serum copper is there any evidence of the elevation of the serum copper in Bantu who exhibit gynecomastia?

WALKER The estrogen levels of our Bantu subjects with gynecomastia have been compared with those of not affected Bantu subjects of the same age groups but not of the same physique As already related according to Dr Bloomberg mean values do not differ significantly among the Bantu with and without gynecomastia As far as I am aware a correlation between urinary estrogens and serum cholesterol level has not been claimed but judging from recent local information I am sure that such a correlation obtains at least among differing population groups In relation to copper Dr Theron at Pretoria has found that serum copper concentration in Bantu adults male and female is significantly higher compared with corresponding white groups

MARMORSTON I wonder if Dr Walker would be kind enough to clarify again the estrogen excretion levels in the two groups of patients As I understand it the studies on the young white and the Bantu are over all studies made in normal individuals and in that group the estrogen excretion levels are higher I assume that this is a combination of those patients who have not gynecomastia as well as those who had breast enlargement Is this correct? And the second question is have bioassays on estrogens been done on these patients as well as fractionation studies? And the third question is have estrogen excretion levels been measured in the cerebral group of patients? I ask this last question because we have measured the estrogen levels in a group of approximately 95 white male subjects and 45 female subjects and the estrogen levels appear lower as compared with their normal age controls

WALKER Excluding the investigations which myself and collaborators have undertaken I have no information on gynecomastia present in the other groups of Bantu studied but neither incidence nor extent would seem to have evoked comment No bio assays on estrogens have been carried out nor determination of levels in Bantu with cerebral lesions

STAMLER In relation to the diet during the critical weaning period and the possibility that partially irreversible kidney damage might occur on a nutritional basis at this time and it might be related to the eventual development of hypertension are there any data on kidney function in the Bantu?

CHAPTER 29

Thyroid and Estrogen Treatment of Hypercholesterolemia in Man

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There can be little doubt that the circulating lipids are influenced by the physiological and pathological secretions of various endocrine glands Alteration in the concentration of the plasma cholesterol and the plasma phospholipids and in the distribution of cholesterol between the major lipoprotein fractions can be produced by many hormones These hormonal influences have recently been reviewed (1 24 26) and it is clear that both thyroid and estrogenic hormones produce significant lowering of the plasma cholesterol the C/P ratio and the beta lipoprotein cholesterol If it can be shown in man that reduction of hypercholesterolemia is desirable these two groups of hormones may prove to be of therapeutic value With the intense interest during recent years in the development of different methods of lowering hypercholesterolemia it is as well to remember that there is still no evidence that reduction of the levels of the circulating lipids is beneficial in human atherosclerosis The arguments in favor of lowering hypercholesterolemia are based on theoretical deductions and on experimental data derived from other species It is often unwise and misleading to project evidence obtained from other species particularly in the case of hormonal studies into the sphere of human atherosclerosis Nevertheless to assume that the reduction of hypercholesterolemia is desirable in man is a useful working hypothesis which cannot yet be confirmed or refuted Thus it is the object of this paper to consider some of the problems and potentialities of thyroid and estrogenic hormones in the treatment of the hypercholesterolemia associated with human atherosclerosis

THYROID HORMONES

For many years it has been known that thyroid extract lowers the plasma cholesterol and increases the basal oxygen consumption and heart rate in hypothyroid and euthyroid patients (11a 18 34) Thyroxine also reduces the beta lipoproteins (20) and lowers the concentration of certain low density lipoproteins (S_f 12-20) as determined by the ultracentrifuge (31) The increase in basal metabolism produced by thyroxine makes it unsuitable for administration to patients

cellent exercise tolerance developed effort angina and required nitro glycerine tablets for the first time for many months When triiodo thyronine was discontinued the angina improved dramatically The daily oral administration of 100 μg of 3 5-3 triiodo l thyronine to an other group of euthyroid hypercholesterolemic men with coronary disease caused elevation of the basal metabolic rate as well as the ex pected changes in the circulating lipids (8)

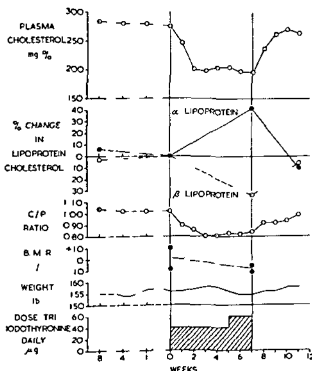


Fig 1 The effects of the oral administration of a small dose of 3 5 3 triiodo-l thyronine to 6 euthyroid men with coronary disease

In Fig 2 the results are shown of the oral administration of increas ing doses of triac from 0 5 to 4 mg daily to 6 hypercholesterolemic eu thyroid men with coronary disease During the administration of less than 3 mg daily there was no significant change in the circulating lipids and lipoproteins but 3 and 4 mg of triac daily produced sig nificant depression of the plasma cholesterol and the beta lipoprotein cholesterol with elevation of the alpha lipoprotein cholesterol There was no significant weight loss and no change in the mean basal meta bolic rate In 2 of these 6 men the basal metabolic rate had risen by

in whom the myocardial blood supply is impaired by coronary atherosclerosis Any increase in the oxygen requirements of the myocardium could be expected to result in myocardial ischemia and lead to the development or worsening of angina Recently several studies have been made of various derivatives of thyroxine in the hope that one of these analogs would lower hypercholesterolemia without elevating basal oxygen consumption

Numerous modifications can be made to the structure of thyroxine and a large number of analogs are theoretically possible Thyroxine can be deiodinated by certain tissues (2) to yield the more thyro active compound 3 5-3 triiodo l thyronine which was first isolated by Gross and Pitt Rivers (14) Further deiodination will yield various isomeric diiodothyronines and monoiodothyronines Apart from these modifications to the diphenyl ether nucleus of the molecule the alanine side chain can undergo oxidative deamination or oxidative decarboxylation Oxidative deamination of thyroxine gives tetraiodothyropyruvic acid which on decarboxylation gives tetraiodothyroacetic acid or "tetrac (15) Similarly triiodothyroacetic acid or "triac (27) can be derived from triiodothyronine Furthermore other halogenated thyronines, such as tetrabromothyronine (16) and tribromothyronine (10) have been shown to have thyroxine like activity in man

In myxedematous patients it has been shown that triac (17 32 33) and tetrac (13) reduced the level of plasma cholesterol without increasing the basal metabolic rate Similarly in euthyroid hypercholesterolemic men with proven coronary disease both 3 5-3 triiodo l thyronine (24) and triac (19 25 33) lowered certain plasma lipids without increasing the metabolic rate This apparent dissociation of actions is illustrated in Figs 1–3 The subjects of these studies were men between the ages of 35 and 55 years They had all sustained myocardial infarction (with electrocardiographic confirmation) more than 9 months before the clinical trial of a thyroid analog and had returned to some form of full time occupation

In Fig 1 the results are shown of the oral administration of 40 μg of 3 5 3 triiodo l thyronine for 5 weeks and then of 60 μg daily for 2 weeks to 6 hypercholesterolemic euthyroid men with coronary disease There was significant depression of the plasma cholesterol the beta lipoprotein cholesterol and the C/P ratio with elevation of the alpha lipoprotein cholesterol but there was no change in weight and none of the 6 men showed any increase in basal metabolic rate The metabolic rates were recorded under basal conditions in hospital on two consecutive mornings before and at the end of the course During this trial of triiodothyronine two men both of whom had previously had an ex

lipoprotein cholesterol and of the C/P ratio with elevation of the alpha lipoprotein cholesterol However after 4 mg had been administered each day for 2 weeks these lipid values returned towards the control levels and although there was further slight depression of the plasma cholesterol when 5 mg were administered the circulating lipids ap

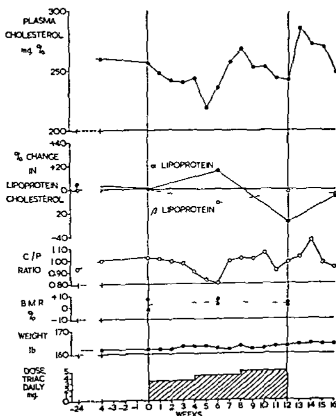


Fig 3 The effects of the oral administration of increasing large doses of triiodothyroacetic acid in 6 euthyroid men with coronary disease (reproduced by courtesy of the *Lancet*)

peared to "escape from the effect of triac There was no loss of weight and the basal metabolic rates of these 6 men were not significantly altered Despite this one of the 6 men experienced an increase in the incidence of effort angina and breathlessness while triac was being administered and regression of these symptoms when triac was discontinued

Thus of the 18 hypercholesterolemic euthyroid men already men

the end of the course and one of these men complained of decreased exercise tolerance and of more frequent episodes of angina of effort In a third man who previously had unimpaired exercise tolerance and no angina the basal metabolic rate actually fell slightly during the course of triac but he developed classical angina of effort several times

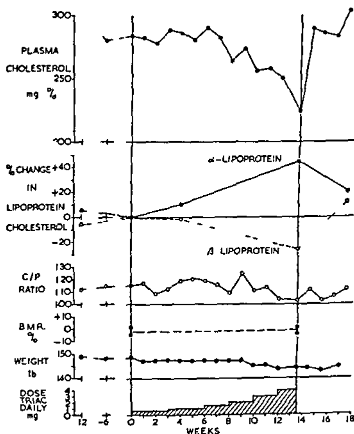


FIG 2 The effects of the oral administration of increasing small doses of tri *iodothyroacetic acid in 6 euthyroid men with coronary disease (reproduced by* courtesy of the *Lancet*)

each day (Fig 4) On withdrawal of triac both these men experienced regression of their symptoms and improvement of their exercise tolerance

In Fig 3 the results are shown of the oral administration of increasing doses of triac from 3 to 5 mg daily to a further 6 hypercholesterolemic euthyroid men with coronary disease A daily dose of 3 or 4 mg of triac caused depression of the plasma cholesterol the beta

results from a discrepancy between the oxygen supply and demand It is readily understandable that an increase in basal metabolic rate should be associated with the development of angina but the occurrence of effort angina in the absence of any increase in basal metabolic rate must be explained The total oxygen consumption of a patient represents the arithmetical sum of the oxygen requirements of all body tissues and it must not be expected that a substance which elevates the basal metabolic rate will influence all tissues uniformly Since the body is composed of tissues of different histological structure and function it would be surprising if they did react toward a metabolic stimulant in the same way For example the administration of desiccated thyroid to euthyroid rats produced a differential response in oxygen uptake between liver kidney diaphragm skeletal muscle and heart tissue (12 35) in the hyperthyroid state produced by desiccated thyroid the greatest increase in metabolic rate of excised tissues occurred in the myocardium Moreover in hypothyroid and euthyroid rats 3-5-3 triodo l thyronine and triac both increased the oxygen consumption of heart tissue to a greater extent than that of liver tissue (3 4 8 9)

The increased frequency of effort angina in patients receiving triac might therefore be related to increased oxygen requirements in the myocardium In other words the dissociation of cholesterol depression from elevation of the basal metabolic rate may be more apparent than real and it could depend partly on inadequacy of the basal metabolic rate measurement to reflect a selective increase in the oxygen requirements of a comparatively small tissue In addition to the increased incidence of pain depression of the plasma cholesterol could not be maintained over long periods even when the dosage of 3 5-3 triodo-l thyronine and triac was increased It is concluded therefore that at present there is no thyroid analog suitable for the long term control of hypercholesterolemia in patients with clinical coronary disease If some thyroid analog is found to lower hypercholesterolemia without causing any selective increase in myocardial oxygen requirements it will have obvious therapeutic potentialities

Estrogenic Hormones

In hypercholesterolemic men with coronary atherosclerosis estrogens lower the plasma cholesterol and beta lipoprotein cholesterol and elevate the plasma phospholipids and alpha lipoprotein cholesterol (5 7 22 30) This action of estrogens is true for synthetic estrogens as well as for estradiol estrone and estriol (23) Ethinyl estradiol and Premarin are probably used more widely than other estrogens to lower hypercholesterolemia The results of the administration of various doses of

tioned 4 experienced reduction of their exercise tolerance on account of effort angina and breathlessness during the administration of 3 5-3 triiodo l thyronine or triac Similar aggravation of effort angina has been observed in 3 patients receiving triac for treatment of myxedema None of these 7 patients showed any increase in basal metabolic rate at the time when angina developed in Fig 4 a typical example

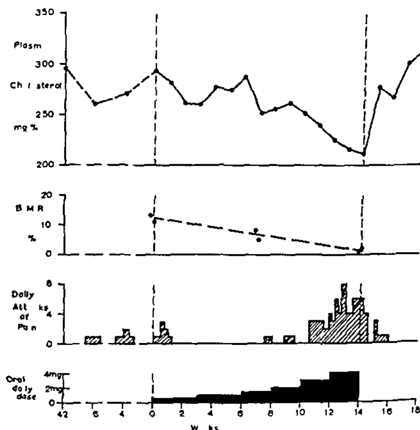


FIG 4 The effects of the oral administration of triiodothyroacetic acid on the plasma cholesterol basal metabolic rate and the incidence of effort angina (A N 51 years)

is shown Similar aggravation of angina without elevation of the basal metabolic rate has been reported in 2 myxedematous subjects by Mackay *et al* (19) Although there was no increase in the incidence of angina in a series of 10 cases reported by Menzies and Cooper (21) one man who had experienced angina for the previous 1½ years died from a myo cardial infarct on the 17th day of treatment with triac

It is widely believed that anginal pain is anoxemic in nature and

ence in the degree or the rate of the depression of plasma cholesterol or of the C/P ratio whether ethinyl estradiol was administered at a level of 200 μg daily or 1 mg daily or increased gradually from 200 μg to 1 mg daily The plasma cholesterol and C/P ratio were reduced in only 7 out of 12 men who received a daily dose of 100 μg of ethinyl estradiol this is regarded as a suboptimal dose level

Of 150 hypercholesterolemic men who have received ethinyl estradiol in varying doses the majority have developed gynecomastia or depression of libido (Table I) Attempts to minimize these feminizing effects by the simultaneous administration of androgens or progestins have not

TABLE I
THE INCIDENCE OF FEMINIZING EFFECTS IN 150 MEN WHO RECEIVED 200 μg OF ETHINYL ESTRADIOL DAILY FOR LONGER THAN 3 MONTHS

Feminizing feature	Men affected (%)
Gynecomastia	98
Depression of libido	73
Loss of libido	42
Testicular pain	3
Penile irritation	3

been successful (6 23) Not only did methyltestosterone fail to relieve the feminization but it counteracted the depression of the circulating lipids produced by ethinyl estradiol Although these feminizing side effects are often quite well tolerated it is obviously desirable to study weakly estrogenic compounds in the hope that it will be possible to dissociate the cholesterol lowering action of estrogens from their feminizing action Various estrogen analogs have been investigated but so far achievement of this dissociation has not been successful

The administration of 3 methoxy 16 methyl estratriene 16 17 diol (Manvene) which is a derivative of estriol has been somewhat disappointing Although in a short term study Robinson *et al* (29) did not observe any feminizing effects in 7 out of 12 of their patients in whom the plasma cholesterol was lowered by a dose of 50 mg daily Davis *et al* (11) indicated that over a longer period the majority of their patients developed feminizing effects at doses of 10 to 20 mg of Manvene daily In our small experience of the administration of Manvene to 6 hypercholesterolemic men with coronary disease (Fig 7) 2 of these men showed depression of the plasma cholesterol when 5 mg were administered daily and 2 other men had reduction of their hypercholesterolemia at a level of 10 mg daily Two of these 4 men developed mild gynecomastia and in the remaining 2 men Manvene

ethinyl estradiol have already been reported (23) and these studies suggested that 200 μg daily is the optimum dose for most men There was no significant change in the hematocrit at this dose level A dose larger than this is not more effective in lowering the circulating lipids and is probably a disadvantage since feminizing effects are more often encountered These comparative dose studies have been elaborated (Figs 5 and 6) and it can be seen that there was no significant differ

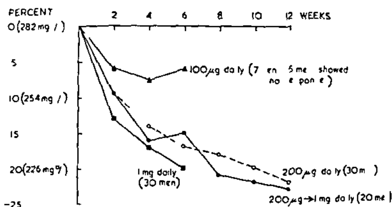


Fig 5 The mean per cent fall in the plasma cholesterol during the oral administration of various doses of ethinyl estradiol

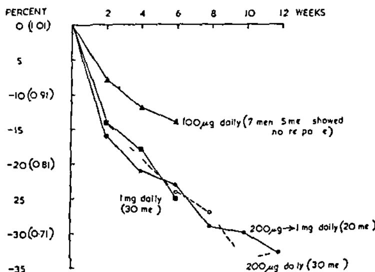


Fig 6 The mean per cent fall in the C/P ratio during the oral administration of various doses of ethinyl estradiol

to their date of discharge None of these men had sustained more than one myocardial infarct and this infarct occurred between 6 and 8 weeks before the start of estrogen therapy Men with a diastolic blood pressure of more than 100 at rest with diabetes or myxedema were excluded Men who had experienced effort angina for more than

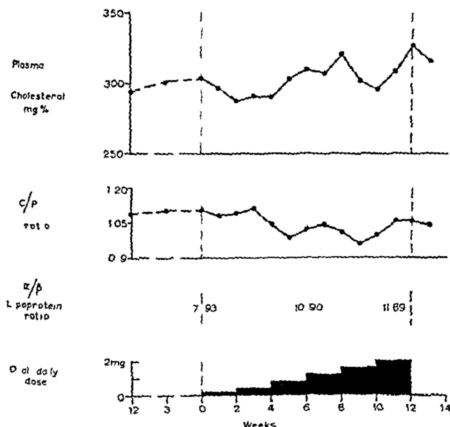


Fig 8 The effects of the oral administration of 1 methyl 17 ethinyl estradiol to 6 men with coronary disease

6 months prior to the infarct or who had developed congestive failure during the convalescent period after the infarct were also excluded from the study Patients who weighed more than 20 lb above the standard weight for their height and age were excluded and no specific dietetic instructions were given to any of the 100 men of this study The men attended hospital once every 3 months during the first year of the study and thereafter they attended every 6 months At each visit when they were seen and examined by the same physician the blood pressure

appeared to be inactive Similarly the administration of 1 methyl 17 ethinyl estradiol and of 4 methyl estratriene 1 ol 17 one has been disappointing (Figs 8 and 9) since both preparations were inactive in respect of depression of the circulating lipids feminization was not produced in any of the 12 hypercholesterolemic men who received these substances If some estrogen analog is found to lower hypercholesterol

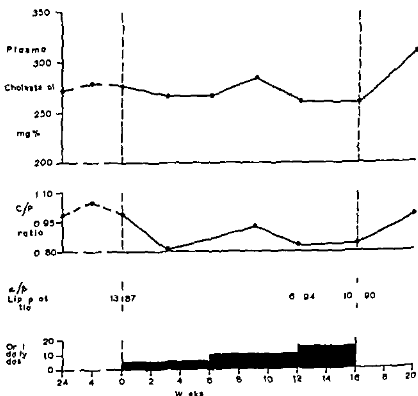
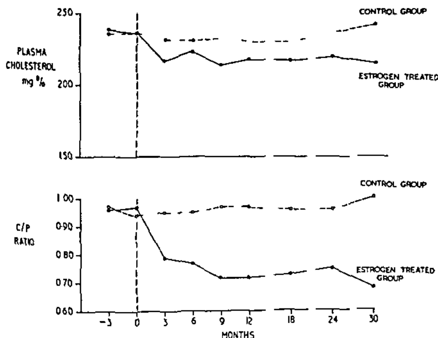


Fig 7 The effects of the oral administration of 3 methoxy 16-methylestratriene 16 17 diol (Manvene) to 6 men with coronary disease

emia without causing feminization it will have obvious therapeutic potentialities

Despite the feminizing effects of ethinyl estradiol this estrogen has been administered to 50 men and has been well tolerated over periods up to 4 years These 50 men were matched with 50 other male patients to whom inert tablets of identical size and shape were administered These 100 men whose ages ranged between 35 and 60 years were discharged consecutively from a male ward in the Edinburgh Royal Infirmary and alternate patients received ethinyl estradiol according

who died from carcinoma of the prostate after receiving large doses of estrogens showed less coronary atherosclerosis than a comparable control group (28) However it is not yet known whether correction of hyper cholesterolemia in man results in regression of atherosclerotic lesions nor is it known whether inhibition of atherogenesis can improve the prognosis once the typical features of atherosclerosis have developed This observation that estrogens did not influence morbidity and mortal



NUMBER OF CASES IN

(1) CONTROL GROUP	50	50	46	45	43	40	40
(2) ESTROGEN TREATED GROUP	50	50	47	45	44	42	38

FIG 10 The effect of the long term administration of ethinyl estradiol on the plasma cholesterol and C/P ratio

ity despite continued reduction of hypercholesterolemia and the C/P ratio is disappointing although it may be important if confirmed It might be argued that estrogens have worsened the prognosis through some action other than on cholesterol metabolism and that these results do not invalidate the hypothesis that it is desirable to induce hyper cholesterolemia The study here reported is small and must continue for at least 5 years before any definite conclusions can be drawn

and weight were recorded and a specimen was taken for plasma lipid analysis and at every alternate visit an electrocardiogram was recorded

The first 2½ years of this study have now been completed it is clear from Fig 10 that ethinyl estradiol reduced the plasma cholesterol throughout this period The greater effect on the C/P ratio emphasizes that estrogens cause elevation of the phospholipids as well as depression of the plasma cholesterol During these 2½ years the majority of the

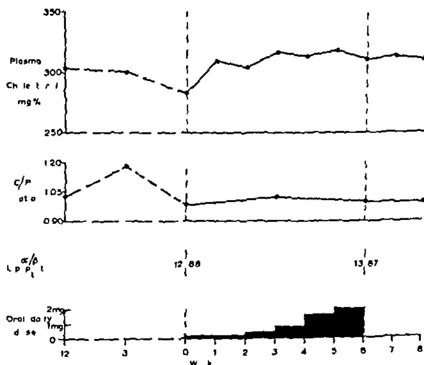


Fig 9 The effects of the oral administration of 4 methyl estratriene 1-ol 17-one to 6 men with coronary disease

men received 200 μg of ethinyl estradiol each day but it was necessary after a period of 9 months to increase this dose to 300 μg in 5 of the men in order to maintain depression of the plasma cholesterol The details of mortality and morbidity in these 100 men during this 2½ year study are shown in Tables II and III It is apparent that so far there is no significant difference between the two groups in respect either of mortality or morbidity

In addition to the very considerable experimental evidence that estrogens may cause regression of atheromatous lesions it has been suggested that such regression may also occur in man autopsies of men

basal metabolic rate Thus at present these analogs are unsuitable for the treatment of hypercholesterolemia in man

Most estrogens cause depression of hypercholesterolemia in man but significant depression is frequently associated with feminization So far it has not been possible to dissociate the cholesterol lowering action of estrogens from this feminizing action and thus estrogens are not suitable for the routine treatment of hypercholesterolemia in man Although estrogens produced constant depression of hypercholesterolemia and the C/P ratio in 50 men during a 2½ year period there was no significant improvement in morbidity or mortality from coronary disease when contrasted with a comparable control group

As yet there is no evidence that reduction of hypercholesterolemia in man is associated with inhibition or regression of the atherosclerotic process It is therefore essential to assess any potentially satisfactory therapeutic regime in terms of morbidity and mortality rather than in its ability to lower plasma cholesterol levels

ACKNOWLEDGMENTS

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REFERENCES

1 Adlersberg D *Am J Med* **23** 769 (1956)
2 Albright E C Larson F C and Tust R H *Proc Soc Exptl Biol Med* **86** 137 (1954)
3 Barker S B *Endocrinology* **59** 548 (1956)
4 Barker S B and Lewis W J *Proc Soc Exptl Biol Med* **91** 650 (1956)
5 Barr D P *Circulation* **8** 641 (1953)
6 Barr D P *J Chronic Diseases* **1** 63 (1955)
7 Barr D P Russ E M and Eder H A *Trans Assoc Am Physicians* **65** 102 (1952)
8 Boyd G S and Oliver M F *Bull schweiz Akad med Wiss* **13** 384 (1957)
9 Boyd G S and Oliver M F *Proc Conf on Hormones and Atherosclerosis* 1958 this volume
10 Compston N and Pitt Rivers R *Lancet* i 22 (1956)
11 Davis F W Jr Scarborough W R Mason R E Singewald M L and Baker B M *Am J Med Sci* **235** 50 (1958)
11a Gildea E F Man E B and Peters J P *J Clin Invest* **18** 739 (1939)
12 Gordon E S and Heming A E *Endocrinology* **34** 353 (1944)
13 Goolden A W G *Lancet* i 891 (1956)
14 Gross J and Pitt Rivers R *Vitamins and Hormones* **11** 159 (1953)
15 Harington C R and Pitt Rivers R *Biochem J* **50** 438 (1952)
16 Lerman J and Harington C R *J Clin Endocrinol* **9** 1099 (1949)
17 Lerman J and Pitt Rivers R *J Clin Endocrinol and Metabolism* **15** 653 (1955)
18 Lévy Max and Lévy M *Bull acad méd (Paris)* **105** 666 (1931)
19 Mackay I R Goble A J and Sparkes F *Med J Australia* i 571 (1957)

TABLE II

COMPLETED 2½ YEAR STUDY OF 50 MEN RECEIVING ETHINYL ESTRADIOL AND 50 MEN RECEIVING INERT TABLETS

	Estrogen group (number of patients)	Control group (number of patients)
Morbidity		
Myocardial infarct	5	5
Acute insufficiency (prolonged pain ECG negative)	2	2
Cerebrovascular incident	3	1
Pulmonary infarct / Thrombophlebitis	5	1
Mortality		
Myocardial infarct or sudden death	8	6
Dissecting aneurysm	1	0
Bronchial carcinoma	0	1
Discontinued		
Long term anticoagulants	1	2
Breast abscess	1	0
Uncooperative	1	1

TABLE III

THE DISTRIBUTION OF SECOND MYOCARDIAL INFARCTS IN RELATION TO THE ADMINISTRATION OF ESTROGEN AND INERT TABLETS

Time after starting estrogen or inert tablets (months)	Nonfatal		Fatal	
	Estrogen group	Control group	Estrogen group	Control group
0–3	0	1	2	1
3–6	1	0	1	0
6–9	0	2	0	1
9–12	0	1	0	1
12–18	3	0	1	0
18–24	1	1	2	2
24–30	0	0	2	0

SUMMARY

Although various thyroid analogs produced depression of hyper cholesterolemia without apparently elevating the basal metabolic rate this cholesterol lowering action could not long be maintained without increasing the dose of the analog up to a level which elevated metabolic requirements Moreover the administration of these analogs was associated with an increased incidence of effort angina and there is experimental evidence that they can increase selectively the myocardial oxygen requirements without elevating the gross measurement of the

duction in serum cholesterol levels without increase in the BMR has often been credited with stimulating interest in the possible development of thyroid analogs which would be hypocholesterolemic in euthyroid subjects in doses which would not increase the BMR I would like to set the record straight and point out that the late Dr John P Peters many years ago indicated that the administration of small doses of desiccated thyroid to patients with myxedema occasionally lowered the serum cholesterol level without changing the BMR So much for that point

The second point I would like to make is that in some of the patients receiving triac Dr Oliver stated that the fall in serum cholesterol was not associated with any change in the BMR In one subject at least at the 4 mg per day dose level there was a fall in body weight This seems to me just another evidence of the crudeness of our technique for measuring the BMR

The third point I would like to speak about pertains to the increase in alpha lipoprotein cholesterol We have been interested in the lipid and lipoprotein effects of thyroidal agents in euthyroid subjects and in patients with thyroid dysfunction In euthyroid subjects who are normocholesterolemic to begin with we have not seen an increase in the alpha lipoprotein after triac administration measured either by refractometric methods or by differential preparative ultracentrifugation followed by cholesterol and phospholipid analyses of the thus separated fractions Would you describe your method for lipoprotein determination? Is it a paper chromatographic method? Are you talking about the per cent of stainable lipids found in the alpha fraction or is there an actual increase in alpha lipoprotein as determined say by elution?

OLIVER We use a filter paper electrophoretic method with subsequent elution of cholesterol off the paper and its determination in milligrams per cent We have no experience of administration of these analogs to normocholesterolemic subjects The lack of rise of alpha lipoprotein cholesterol might be a dose effect since we did not observe such elevation with large doses of these analogs

WALKER Was the extent of gynecomastia much the same in all subjects?

OLIVER It was mu h the same in all subjects and appeared fully developed after about 6 months but it was not like the gynecomastia you see in the Bantu There is far less profuse enlargement of breast tissue in comparison with your patients

STAMLER *Is it possible that the development of angina in patients on triac* without an increase in BMR might also be due to the stimulating effect of thyroid preparations on adrenal medullary secretion with a resultant increase in cardiac metabolism and therefore in cardiac oxygen requirements?

OLIVER This is certainly possible

BOYLE I hate to get back to the clinical aspects of these studies with estrogen but I have had different experience with thromboembolic phenomena and mortality from cardiovascular thrombosis or hemorrhage than in your patients I reported in 1954 at the American Society for the Study of Arteriosclerosis on a 2 year study *on large doses of estrogen* in hypercholesterolemic postcoronary infarction patients and now we have not a large group but a small group that have gone into the sixth year of follow up and those individuals are on 1 mg or more of ethinyl estradiol and have had no such thrombotic complications I might add that in this higher dose response range there are tremendous blood changes as much as 25% drop in the volume of packed red cells a 3-fold decrease in plasma or whole blood viscosity and also a slight prolongation of the separate tube clotting method *in vitro* that might explain the difference in our mortality figures There is another more pronounced hematological change other than on the plasma lipids that occurs

20 Malmros H and Swahn B *Acta Med scand* **145** 361 (1953)
21 Menzies J S and Cooper W F *Med J Australia* i 573 (1957)
22 Oliver M F and Boyd G S *Am Heart J* **47** 348 (1954)
23 Oliver M F and Boyd G S *Circulation* **13** 82 (1956)
24 Oliver M F and Boyd G S *Lancet* ii 1273 (1956)
25 Oliver M F and Boyd G S *Lancet* i 124 (1957)
26 Oliver M F and Boyd G S *Vitamins and Hormones* **16** 147 (1958)
27 Pitt Rivers R *Lancet* ii 234 (1953)
28 Rivin A U and Dimitroff S P *Circulation* **9** 533 (1954)
29 Robinson R W Cohen W D and Higano N *Circulation* **14** 489 (1956)
30 Steiner A, Payson H and Kendall F E *Circulation* **11** 784 (1955)
31 Strisower B Gofman J W Galioni E F Almada A A and Simon A *Metabolism Clin and Exptl* **3** 218 (1954)
32 Trotter W R *Lancet* ii 374 (1955)
33 Trotter W R *Lancet* i 885 (1956)
34 Turner K B and Steiner A *J Clin Invest* **18** 45 (1939)
35 Ullrich W C and Whitehouse W V *Am J Physiol* **171** 407 (1952)

Discussion

BOYLE I have two questions One in your thyroid experiments where on 4 mg of triac you had a fairly marked increase in alpha lipoprotein cholesterol and a marked decrease in beta lipoprotein cholesterol without a concomitant alteration in the cholesterol phospholipid ratio these lipoprotein changes are qualitative ones and the difference in these shifts in alpha beta cholesterol that you see with estrogen in which the cholesterol phospholipid ratio does change to a lower value would suggest thyroid produces a different type qualitatively from estrogen And secondly on the 200 gamma dose of ethinyl estradiol did you notice any pronounced hemo dilution or blood volume changes with decrease in hematocrit red cell counts and hemoglobin and decrease in blood viscosity? Please describe the hematological changes associated with this 200-gamma dose as opposed to the 1 mg dose

OLIVER *There was more consistent and marked depression of the C/P ratio* with estrogen than with thyroid analog administration this is probably partly due to the phospholipid elevating action of estrogens There was no change in the hematocrit at a dose level of 200 μg of ethinyl estradiol but at the 1 mg dose level there was evidence of hemodilution this became apparent at a dose of about 400 μg

EDER *I should like to present some of our data with triac On a dosage of* 2 mg per day there were no significant reductions in total serum cholesterol and there were no consistent changes in oxygen consumption On 4 mg per day the mean decrease in serum cholesterol in our group was 16% and we found consistent elevation in oxygen consumption of about 10% There were however occasional patients in whom serum cholesterol did decrease but in whom increases in oxygen consumption did not occur

We would conclude that our experience was somewhat different from that of Drs Oliver and Boyd in that basal oxygen consumption did increase more consistently

OLIVER It may be the case that elevation in BMR (basal metabolic rate) will be observed more readily if the initial dose is fairly high *Starting at 3 mg* and increasing fairly quickly up to 5 or 6 mg may be associated with elevation of the BMR at a dose level of 4 or 5 mg but with a gradual build up elevation of the BMR *is not so apparent*

FURMAN The observation of Lerman and Pitt Rivers cited by Dr Oliver in which the exhibition of small doses of triac to hypothyroid subjects resulted in re-

was that they had in their pulmonary arteries atheroma in no way different in degree according to the pathologist than that which might have been seen in patients with mitral valvular disease in whom hypothyroidism had not been induced We concluded that hypothyroidism in man with its attendant hypercholesterolemia did not of necessity result in coronary atherosclerosis

KATZ I would like to make a comment on the development of angina pectoris and I am glad that I follow Dr Freedberg because I think that the problem of the angina is different from the problem of myocardial infarction and atherosclerosis I am not going to deal with the latter two

In some animal work done with a special preparation we have found that in the dog a good index of the oxygen consumption of the myocardium is the product of heart rate and mean blood pressure—measured planimetrically (but I imagine that the arithmetic average of systolic and diastolic blood pressures would do) I put it to you that this product might be a useful index of cardiac oxygen consumption in man and probably as good as the data by coronary sinus catheterization that has become so popular Now since blood pressure does not change much ordinarily in man heart rate alone in your patients on thyroid and thyroid extracts might give you an idea of the rate of oxygen consumption per unit of time Further since coronary flow is limited under conditions of coronary artery disease coronary flow might not keep pace and coronary insufficiency and angina might result

In the second place basal metabolism does not give a good idea of the oxygen cost of the daily activity of these people Hyperthyroid people are hyperdynamic so that the 24 hour-day caloric value could be higher even though the basal metabolism did not change

I have a question directed to Dr Boyle about his comments I would like to have in the record how many patients he studied how well they were matched not just the vague statement that he has studied a small group that he has followed for many years It seems to me that we are now in a period where several groups are doing elaborate studies The details of how such studies are done are important in evaluating the conclusions of the authors

BOYLE I have done no age matching controls in this series It is difficult as you know to get patients to take feminizing doses of estrogen for prolonged periods of time and I have currently 5 patients that I have followed since the latter part of 1952 and who are still on estrogen I lost a large percentage of the group of about 40 patients when I left Bethesda and moved to South Carolina and the others that I left up there have discontinued the drug I have resumed another series which is now an 18–24 month series in Charleston so that this interruption is making it a little difficult to evaluate the number of patients totally treated with estrogens chronically but it is a little over 100 The length of time varies anywhere from 3 months up to as much as 6 years I have 2 hyperlipemic patients that have been on this drug one for 6 years and one for 4 years one of which is diabetic his diabetes and hyperlipemia are completely normal as long as he is on this large dose of estrogen I am basing my decreased incidence of deaths from cardiovascular thrombosis on what my friends from the Metropolitan Life Insurance Company tell me is the life expectancy of a patient who is treated post myocardial infarction I think you were in attendance at the A S S A meeting in 1954 and I do not have with me the statistical data you want now and you should have seen then

OLIVER Dr Boyle s group is not really comparable with ours and it might be erroneous to contrast the results It is probably wiser to control this type of morbidity and mortality study by a comparable group than by the known expectancy of life following a myocardial infarct The prognosis following myocardial infarction is too unpredictable for use as a control

with the higher dose These plethoric male coronary patients while on estrogen will have a similar blood pattern hematologically to that of a young menstruating female

ROSENMAN I would like to ask Dr Oliver if there were any findings of interest at the time of autopsy of those treated patients who died and secondly if the cause of death in these patients was coronary thrombosis

OLIVER There were 8 deaths in the estrogen treated group and we have had 3 autopsies As you know this disease often ends with sudden death at home or when the patient is at work and I think we were fortunate to have even 3 autopsies out of 8 deaths I would not like to make any comment at all about autopsy findings in so few cases

MARMORSTON I wonder if Dr Oliver would clarify again whether it is correct that all of these men received 200 gamma of ethinyl estradiol throughout the entire period of the experiment What was the age of these men and how were they selected for treatment? Were they randomized? I believe that Dr Oliver intends to continue to treat these same men for a long period of time is this correct?

OLIVER Of the 50 men 45 have received 200 µg of ethinyl estradiol throughout but in the remaining 5 men it was necessary to increase the daily dose to 300 µg in order to maintain the lipid depression thus the majority received 200 µg daily The age of both groups was comparable and ranged between 35 and 60 years Alternate patients received ethinyl estradiol and inert tablets After exclusion of hypertensive obese myxedematous and diabetic patients 100 consecutive patients were chosen for the study We will continue this study for at least 5 years

FREEDBERG I would like to ask Dr Oliver a question and then make a comment First was there any difference in the development of angina in the two groups?

OLIVER No

FREEDBERG What percentage developed angina?

OLIVER The assessment of angina in these men was rather difficult in so far as both groups had less angina when they started treatment Thereafter most men in both groups had angina sometime during the 2½ years but there was no significant difference between the two groups

FREEDBERG The comment I would like to make is with reference to the experience which we have had in which we have induced hypothyroidism over the past 25 years in over 200 patients with severe intractable angina pectoris due to coronary artery disease Originally this was accomplished by surgical total thyroidectomy and in the past 12 years with I^{131} These patients are maintained at hypothyroid levels with a BMR of −20 to −25% with daily doses of desiccated thyroid of 15 to 20 mg The PBI levels are very low the serum cholesterol levels are high and the β lipoprotein levels are high Many continue to have mild symptoms of hypothyroidism In the patients with angina pectoris due to coronary artery disease pathologists cannot tell us whether these individuals have more coronary artery disease or less than control patients There is a group of these hypothyroid patients that we have in whom coronary artery disease was not expected these are patients with mitral or other valvular disease who have angina pectoris and congestive failure In 10 of these patients who have died 1 to 13 years (average 7 years) after the induction of hypothyroidism careful postmortem examinations have been made These patients were 35 to 65 years of age 6 were male and 4 female In the patients examined at the Beth Israel Hospital, the coronary arteries were studied with the injection dissection technique of Dr Monroe Schlesinger Their cerebral arteries the aortae the peripheral arteries as well as the coronaries showed a remarkable freedom from atheroma They showed an absence of intercoronary arterial collateral circulation The interesting thing about some of these patients

Interim Report on Clinical Experiences with Long Term Estrogen Administration to Middle Aged Men with Coronary Heart Disease

J STAMLER [1] R PICK, L N KATZ A PICK, AND B M KAPLAN

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I shall first summarize very briefly the background of scientific findings which led us to undertake a long term study on the possible therapeutic efficacy of estrogens in the treatment of human coronary disease Dr Ruth Pick has already presented the basic epidemiologic and clinical facts on the marked insusceptibility of middle aged women to coronary disease—facts which inevitably led to the hypothesis that estrogenic secretion may be a key factor protecting the female sex She has also reviewed our experimental findings demonstrating that estrogens both prophylactically inhibit and therapeutically reverse atherosclerotic plaques in the coronary vessels of chickens fed a high fat, high cholesterol diet When this study was initiated late in 1952 data were available from other laboratories demonstrating a sex difference in serum cholesterol lipid lipoprotein levels and demonstrating that estrogens convert male cholesterol lipid lipoprotein patterns into female patterns (1 2, 12) As this conference has already heard these observations have been extensively confirmed and elaborated In the intervening years additional clinical research has yielded considerable further evidence indicating that estrogens may protect human beings from coronary disease (3 8 11 18)—evidence that has been reviewed in detail elsewhere (6 16) None of these studies attempted to determine whether estrogens are effective in the treatment of coronary disease

It was deemed essential to attempt such an evaluation—focusing not on estrogen effects on plasma lipids lipoproteins but on the critical question Will estrogen therapy reduce the recurrence rate of myocardial infarction and prolong the life span in patients with clinical coronary heart disease? This then has been a study—by necessity a long term investigation—of the therapy or (if you will) of the secondary prophylaxis of coronary disease with estrogens

With this as background let us now proceed to our study proper its detailed objectives problems and findings to date At the onset let us

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BOYLE I agree that the number in my series is much too small for this interpretation in contrast to a large group However I think that another area in which these two studies should be correlated is the dose response As far as the clinical symptoms of gynecomastia and other side effects are concerned we see similar effects but they are more rapid with the larger dose but in no degree more severe It takes a longer period of time on a smaller dose but this big problem with hematological changes is an entirely different dose response and I think in the long run will make a fairly sizable difference in what happens to these individuals I think side effects of estrogen on hematological characteristics blood viscosity and coagulation are as important as lipid effect in thrombosis

ADLERSBERG I would just like to ask a brief question What is your definition of hypercholesterolemia? In some of your slides I noticed cholesterol levels of 250 mg % or so In view of our recent studies we became very conservative in using the term hypercholesterolemia because of the change in cholesterol levels and other lipid levels e g phospholipids with age and sex We decided to use the upper 5% levels of a given age and sex group for defining hypercholesterolemia

OLIVER Thank you Dr Adlersberg for bringing up that point In Britain we believe that the plasma cholesterol level is a little lower than in the United States Following the study some years ago of 250 healthy subjects from 25 to 75 years we concluded that the range for our laboratory in Edinburgh was between 160 and 220 mg % Since there was no significant rise with increasing age we have defined hypercholesterolemia as greater than 250 mg % This figure represents the addition of two standard deviations to the mean of this healthy group

ADLERSBERG Using what technique?

OLIVER The Sperry and Webb modification of the Sperry Schoenheimer technique

STAMLER I would just like to add a word to this I think one of the most important problems we face is the problem of the evaluation of normalcy If one takes the approach of examining clinically healthy Americans one invariably finds a mean serum cholesterol level of about 240 mg % in middle aged males with about 20 to 25% of subjects having values above 270 mg % If one defines as normal people who are clinically free of disease and as normal biochemical levels the values found in such persons then *ipso facto* these are normal values If one goes beyond such a single measurement and takes into consideration the emerging data on the correlation between level of cholesterol and risk of developing myocardial infarction (from the findings of the Cooperative Lipoprotein Study the Framingham Heart Study and other epidemiologic studies) one must conclude that the upper limits of normal for serum cholesterol should be set at 225 or 210 or even 200 mg % This latter approach introduces a time dimension into considerations of standards of normalcy It poses the problem of the prognostic or risk significance of given cholesterol levels It introduces the consideration What is an optimal serum cholesterol level for optimal freedom from atherosclerotic disease over an optimal life span?

ADLERSBERG I would like to say that one cannot compare as a whole the population in Edinburgh with that in New York City Chicago or Johannesburg in regard to circulating normal" lipids One has to establish normal values for a specific population group living under the prevailing climatic social economic and nutritional conditions whether ethnic factors play a part nobody knows I believe that for purposes of determining what is normal and abnormal in lipid levels one cannot simply refer to a population group studied by somebody else under completely different conditions

This selection of mortality as an end point in the evaluation of therapy for coronary heart disease was a critically important decision Of course we have been following a multiplicity of other phenomena in the treated and placebo patients—chest pain electrocardiographic patterns serum cholesterol lipid lipoprotein levels patterns of employ ment work physical activity familial relationships psychological ad justment etc For therapeutic evaluation however the decisive critical end point was set as the effect of estrogen on survival rate Recurrence rate of nonfatal myocardial infarction was designated a second auxiliary end point It was further decided to evaluate the death rates from all causes from cardiovascular renal diseases and from recurrent myocardial infarction

Another problem was that of estrogen dosage In essence a decision had to be made between two alternatives—to study the effects of low dosage or high dosage estrogen therapy The latter was elected The objective was therefore set of giving a high enough dosage of hormone to obtain typical estrogen effects on serum lipid lipoprotein patterns and secondary sex characteristics

The original design provided that all patients should be men under 50 with a single proved myocardial infarction within the preceding 12 month period uncomplicated by hypertension other heart or systemic disease e g diabetes hepatic thyroid or renal disease These provisos soon proved to be quite unrealistic There was a paucity of such "pure" cases In addition another problem arose Patients were being obtained by referral from the medical staff of the Michael Reese Hospital Many of these patients did not meet the aforementioned rigid criteria Some had had more than one infarct a few were cases of chronic coronary insufficiency with or without angina pectoris others had experienced their myocardial infarction more than a year previously some had diabetes or hypertension Since it was essential both to build up an ade quate case series and to maintain rapport with the physicians at the Michael Reese Hospital it was decided to accept these "complicated" patients and to randomize them separate and apart from the "pure" cases The study therefore involves Series" ("pure) and "Off series" ("complicated") patients with both Series and Off series divided into placebo and treated groups—i e four groups in all However patients with congestive heart failure persisting after recovery from the acute attack were excluded from the study The total case load of 276 patients was ultimately achieved only through the splendid cooperation of the Departments of Medicine of several Veterans Administration Hospitals in the Chicago area and throughout the Midwest (see Acknowledg ments)

emphasize that this is an interim report it is not possible at this juncture to give a definitive answer as to whether estrogens are or are not efficacious in the treatment of coronary disease

Two broad objectives were set in launching this study late in 1952 (*1*) to evaluate estrogen therapy (*2*) since it was imperative to have a control (placebo) group to utilize the control group to extend understanding of the natural history of coronary disease At the onset certain major questions of design presented themselves *As the study evolved* additional problems of design arose Our initial decisions were to evaluate estrogen therapy in middle aged males (under age 50) with clinical coronary heart disease i e with definite myocardial infarction[2] Our initial design involved three groups a placebo group an estrogen group and an estrogen androgen group During the first year of the study (1953) the estrogen androgen group was abandoned for several reasons This combination in the initial dosage utilized (1 25 mg of mixed conjugated equine estrogens plus 10 0 mg of methyltestosterone orally) was found to have estrogenic effects on the secondary sex characteristics Thus feminizing effects were not avoided Further the estrogen androgen combination yielded androgenic effects on the serum lipids lipoproteins whereas the objective was to convert the male into the female pattern In addition we became increasingly concerned with the possible hepato toxic effects of long term androgen administration *Finally it soon* became clear that it was no simple matter to acquire the prerequisite number of patients for two groups let alone for three groups For all these reasons the estrogen androgen group was put on estrogens only As the data are presented the consequence of this will be apparent in that the estrogen group is somewhat larger than the placebo group In all other respects, patients serially entering the study were randomly assigned to the two groups on a double blind basis

From the beginning a definite minimum therapeutic objective was set *It was agreed that estrogen treatment* with dosages producing feminizing side effects would not be justified in coronary disease unless a well controlled study demonstrated that the mortality rate in the estrogen group was at least one half or preferably one third of that in the control group Of course this mortality experience—in order to be meaningful in a disease with a natural history such as coronary disease—had to be accrued over a several year period And of course this medically significant result (if it were obtained) also had to be statistically significant with tolerable confidence limits

2 *Ultimately the placebo and treated groups* each contained a few cases of coronary heart disease manifesting itself as angina pectoris or chronic coronary insufficiency without proved infarction

Two other cardinal problems in such a long term study are evaluating adherence to the drug and evaluating lost patients (patients who drop out of the study) With respect to adherence two objective criteria were fortunately utilizable—development in patients on high dosage estrogens of gynecomastia and of feminine cholesterol lipid lipoprotein patterns particularly a fall in cholesterol/phospholipid (C/P) ratio and a rise in alpha lipoproteins These objective criteria permitted an evaluation of adherence to the drug regimen by individual patients and an effective challenge to "cheaters" protesting their innocence The statistical handling of lost patients is discussed below in the course of presenting the data to date With this information on design as background the data as of March 1958 follow

In accord with the experience of many other investigators studying middle aged males with coronary heart disease our patients as a group exhibited control pretreatment levels of serum cholesterol and beta lipoproteins that were high compared with those for age matched American males free of clinical coronary disease (Figs 1 and 2) The differences are more gross when the comparison is made with Central Americans Bantu or Navajos (4–7 10 13–15 17) A discussion of the theoretical significance of these findings is beyond the scope of this paper (cf 6 14 15)

The pretreatment values of the four basic groups for serum total cholesterol phospholipid C/P ratio and lipoproteins are summarized in Figs 3 and 4 Note the similarity of the findings on the four groups They are indeed highly comparable with respect to these parameters Other control data bearing upon this problem of matching of paired groups treated and placebo are presented in subsequent figures and tables

At this juncture before proceeding to the effects of estrogen therapy a word may be in order concerning an intriguing theoretical problem posed by the accumulated cholesterol lipoprotein data Among the placebo patients on whom multiple cholesterol lipoprotein determinations have been accomplished at intervals over several years a few consistently exhibit alpha lipoprotein concentrations well over 200 mg % i e they have alpha lipoprotein levels in the female range considerably higher than those of most males (patients ## 11 7631 11 7652 11 7688 12 222 11 7676 Table I—cf Figs 2 and 4) These alpha lipoproteins in placebo patients are in the range observed in the men on large dosage estrogen therapy It has been implied that such "female lipoprotein patterns are "good" in terms of protection against coronary disease Yet these men have suffered a myocardial infarction prior to age 50 With respect to cholesterol levels a few placebo patients consistently

A major concern was to do everything conceivable to assure maximum matching and comparability of the paired groups The distinction between Series and Off series and the randomized assignment of patients to a group on a double blind basis aimed to achieve this purpose Extensive auxiliary data medical and sociological were collected on each patient and analyzed on a group basis to assess comparability of the groups and possible sources of bias Information was obtained on race nationality religion occupation height and weight therapeutic regimen, including diet and anticoagulant therapy both of which we attempted (with success) to hold to a minimum in these patients Determinations were also made of pretreatment serum cholesterol phospholipid, and lipoprotein levels In addition an assessment was made of the nature of the acute episode of myocardial infarction Based on absence or presence of complications during the initial 4 to 6 weeks following onset of the attack patients were classified into good or poor risk i e patients whose acute infarction was uncomplicated by shock friction rub arrhythmia congestive heart failure extension of the infarct or other major complications were classified as good risk patients Those with complications were classified as poor risk All patients with more than one infarction were also classified poor risk Inadequate information was available concerning the acute episode in a few patients those were designated unknown risk Clear cut data are now available demonstrating that long term prognosis is related to occurrence of complications during the acute attack Based on these multiple criteria repeated analyses of paired groups and subgroups have generally revealed them to be closely matched and highly comparable (see subsequent figures)

The following other procedures were adopted at the outset a patient with an acute myocardial infarction would not be accepted for estrogen therapy until at least 2 months after onset of the attack—i e a post infarction treatment and convalescent period of at least 2 months Finally in order to allow a time period for the effects of hormonal therapy to become operative the decision was made—a decision requiring re evaluation for reasons the data will clarify—that any episode occurring during the first 2 months of treatment would not be counted as a statistically meaningful event Since this decision had been made for the estrogen treated group, it obviously had to be applied to the placebo group Hence *based on this initial decision no patient was scheduled to become a statistical member of the study—so to speak—until at least 4 months after onset of his acute attack* However the occurrence of certain unforeseen events during the initial 2 months of *high dosage hormone administration* made it essential to evaluate this period carefully (see below)

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exhibit serum cholesterol levels under 200 mg % (patients ## 11 7676 12 158 12 200 12 210 Table I—cf Figs 1 and 3) Patient # 11 7676 has uniformly had both a low serum cholesterol and a high alpha lipo protein level These are exceptions to the general rule that clinical coronary disease in men under 50 is associated with hypercholesterol emia It should be emphasized that these "exceptions to the rule are

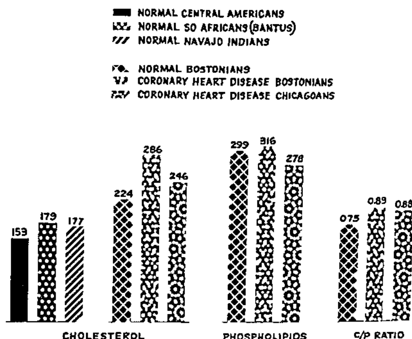


FIG 1 Serum lipids in middle aged normal males and males with coronary heart disease For sources of data on normal Central Americans South African Bantu Navajo Indians and Bostonians see refs 4 5 7 10 13 17 We are in debted to these authors and their publishers for permission to reproduce the data in this and the following figure In this and subsequent figures the numbers at the top of the bars are the actual values where more than one variable is plotted separate scales are used for each variable (in this case each set of 3 bars) in order to facilitate charting

few and far between Yet here they are What about these patients? I present them as fascinating theoretical problems A detailed metabolic and general medical workup of 3 of these patients failed to uncover any abnormalities that would help to account for their having a myocardial infarction prior to age 50 despite low serum cholesterol levels

Now to the matter of estrogen dosage At first a single oral daily tablet of 1 25 mg of mixed conjugated equine estrogens (Premarin) was given The control group of course received a daily placebo tablet

TABLE I

SERUM CHOLESTEROL AND ALPHA LIPOPROTEIN DATA IN PLACEBO PATIENTS CONSISTENTLY EXHIBITING HIGH ALPHA LIPOPROTEIN LEVELS OR LOW CHOLESTEROL LEVELS

Patient number	Number of cholesterol determinations	Serum cholesterol (mg %)	C/P Ratio	Number of lipoprotein determinations	Alpha lipoprotein (mg %)
11 7631	16	265 ± 33*	0 90 ± 0 10	7	235 ± 77
11 7652	17	249 ± 34	0 90 ± 0 11	7	210 ± 81
11 7688	11	250 ± 43	0 90 ± 0 11	3	254 ± 81
12 222	4	317 ± 17	0 99 ± 0 01	2	273 ± 4
11 7676	14	198 ± 16	0 86 ± 0 09	6	213 ± 77
12-158	18	179 ± 24	0 86 ± 0 12	6	135 ± 50
12-200	11	199 ± 15	0 88 ± 0 10	5	140 ± 50
12 210	11	192 ± 66	0 93 ± 0 30	5	140 ± 30

* Standard deviation of the mean

throughout As already indicated our objective was to feminize clinically and to induce typical estrogen effects on cholesterol phospholipids C/P ratio and alpha lipoproteins No such changes were obtained with the lower dosages 1 25 and 2 5 mg/patient/day (Fig 5) They did occur in a high percentage but not in all patients given 4 mg of Premarin per day The initial and maintenance dosage was therefore in June 1954 set at 10 mg All patients previously on lower dosage were put on 10 mg at that time Patients entering the study from June 1954

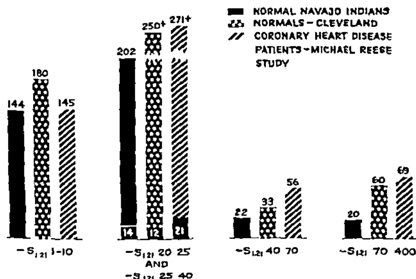


FIG 2 Serum lipoproteins in middle aged normal and coronary heart disease males

on were started on a dosage of 10 mg when assigned to the treated groups This aspect of the evaluation of the study design is emphasized for reasons that will be apparent later

In agreement with the findings of Drs Oliver and Boyd (9) it has been our experience that the earlier patients whose dosage was increased

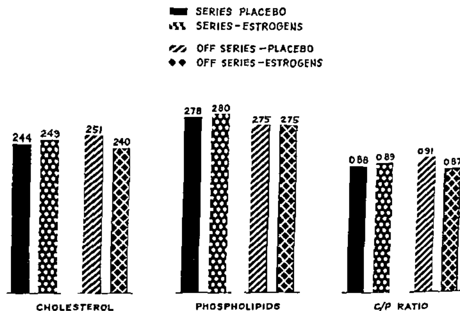


Fig 3 Pretreatment serum lipids

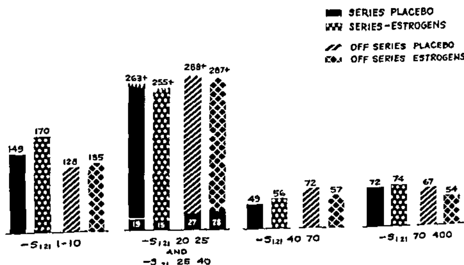


Fig 4 Pretreatment serum lipoproteins

stepwise and the later patients whose dosage was 10 mg from the outset similarly attained typical feminine patterns of C/P ratio and alpha lipoprotein With Premarin sustained reduction in serum cholesterol levels was not consistently observed Some patients manifested a fall in cholesterol others did not with the higher dosage levels of Premarin Phospholipids uniformly rose and tended to remain at higher levels with consequent lowering of the C/P ratio Alpha lipoprotein levels rose markedly and persisted at feminine levels throughout months and years of estrogen administration (Figs 5-7 Table II) Alpha lipoprotein proved to be the most sensitive index of estrogen effect The data on repeat lipoprotein levels at 6 month intervals do not indicate

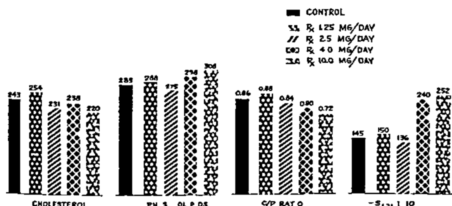


Fig 5 Effects of various estrogen dosages on serum lipids and lipoproteins

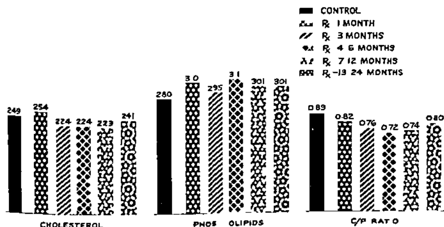


Fig 6 Serial effects of estrogens (10 0 mg/day) on serum lipids—series patients

TABLE II

EFFECTS OF ESTROGEN TREATMENT (10 mg) ON CHOLESTEROL AND LIPOPROTEINS—MICHAEL REESE AND HINES PATIENTS HAVING CORRESPONDING LIPID DATA (DATA AS OF OCTOBER 1957)

$-S_{1\ 21}$	S_f	Control	1 month	2 mo	3 mo	5–7 mo	8–10 mo	12–14 mo	16–20 mo	21–27 mo
1–10	(α)	136	199[a]	228[a]	255	228[a]	248[a]	203[a]	222[a]	240[a]
20–25	1–3	22	15	19	25	19	21	15	14[a]	12[a]
40–70	12–20	55	48	46	48	47	46	55	50	54
70–400	20–100	72	74	57	74	68	68	59	62	78
Cholesterol		243	234	248	201	237	239	230	243	227
α/Cholesterol		0 56	0 88[a]	0 91[a]	1 31[a]	0 96[a]	1 05[a]	0 90[a]	0 97[a]	1 09[a]

[a] Significantly different from control

any tendency for the development of tolerance or adaptation to the hormone

Now to the data on survival The findings on patients entering the study before June 1954 are presented in Table III Altogether there were 93 such patients 37 placebo and 56 treated the latter all receiving hormone at an initial dosage level less than 10 mg The data are presented for several matched pairs of subgroups Since the total number of patients is small these subgroups have been pooled together into

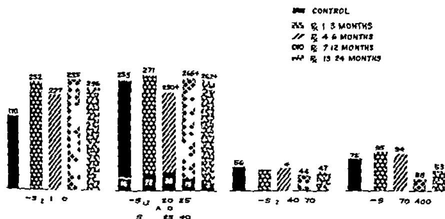


Fig 7 Serial effects of estrogens (10 0 mg/day) on serum lipoproteins—series patients

the two main groups placebo and estrogen treated The data on comparability of these two groups are presented in Table IV They are similar in age height weight and per cent of cases with more than one infarct prior to onset of the study They are closely matched with respect to duration since the first infarct (64 3 and 63 8 months respectively) and duration under study (53 8 and 54 9 months respectively) The estrogen treated group has a lower per cent of Off series patients a lower per cent of poor risk patients and a lower mean serum cholesterol level—differences which may perhaps give the estrogen group a better prognosis As is apparent from Table III the death rate is in fact lower in the estrogen group and in virtually all estrogen subgroups compared with their matched pairs However these results are clearly not statistically significant at this juncture

A slightly larger group of patients—101 in all 40 placebo and 61 estrogen treated—have been under observation for at least 3½ years The data on comparability survival and mortality are presented in Fig 8 These two groups are highly comparable—based on every one of

TABLE III
DEATH DATA AS OF MARCH 1, 1958—PATIENTS ENTERING STUDY BEFORE MAY 31, 1954

Group	N[a]	Total deaths and %	Non CVR deaths and %	CVR deaths in 1st 2 months of treatment and %	Coronary CVR and unknown[b] deaths after 1st 2 months ("valid" deaths) and %
All	93	18(19.4%)	3(3.2%)	1(1.1%)	14(15.7%)
All placebo	37	10(27.0%)	0(0 %)	0(0 %)	10(27.0%)
All estrogen	56	8(14.3%)	3(5.4%)	1(1.8%)	4(7.7%)
Series placebo	20	4(20.0%)	0(0 %)	0(0 %)	4(20.0%)
Series estrogen	36	6(16.7%)	2(5.6%)	1(2.8%)	3(9.1%)
Series placebo good risk	18	4(22.2%)	0(0 %)	0(0 %)	4(22.2%)
Series estrogen good risk	27	3(11.1%)	2(7.4%)	0(0 %)	1(4.0%)
Series placebo poor risk	2	0(0 %)	0(0 %)	0(0 %)	0(0 %)
Series estrogen poor risk	5	2(40.0%)	0(0 %)	1(20.0%)	1(25.0%)
Off series placebo	17	6(35.3%)	0(0 %)	0(0 %)	6(35.3%)
Off series estrogen	20	2(10.0%)	1(5.0%)	0(0 %)	1(5.3%)
Off series placebo good risk	5	0(0 %)	0(0 %)	0(0 %)	0(0 %)
Off series estrogen good risk	11	0(0 %)	0(0 %)	0(0 %)	0(0 %)
Off series placebo poor risk	10	6(60.0%)	0(0 %)	0(0 %)	6(60.0%)
Off series estrogen poor risk	7	2(28.6%)	1(14.3%)	0(0 %)	1(16.7%)
All good risk placebo	23	4(17.4%)	0(0 %)	0(0 %)	4(17.4%)
All good risk estrogen	38	3(7.9%)	2(5.3%)	0(0 %)	1(2.8%)

TABLE III (*continued*)

Group	N[a]	Total deaths and %	Non CVR deaths and %	CVR deaths in 1st 2 months of treatment and %	Coronary CVR and unknown[b] deaths after 1st 2 months ("valid" deaths) and %
All poor risk placebo	12	6(50 0%)	0(0 %)	0(0 %)	6(50 0%)
All poor risk estrogen	12	4(33 3%)	1(8 3%)	1(8 3%)	2(20 0%)

[a] N is the number of patients

All CVR (cardiovascular renal) deaths and deaths during the first 2 mos of treatment subtracted from the denominator as well as the numerator for calculation of per cent valid deaths

All placebo unknown risk — 2 patients no deaths

All estrogen unknown risk — 6 patients 1 death

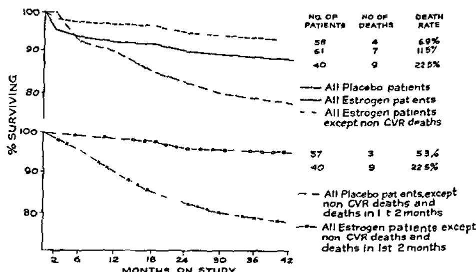
[b] Here and in subsequent figures deaths due to unknown causes are assumed to be coronary or cardiovascular renal deaths

TABLE IV

BASIC INFORMATION ON PATIENTS WHO ENTERED THE STUDY BEFORE MAY 31 1954

	Placebo	Estrogen
Series	54%	64%
Off series	46%	36%
Risk		
Good	62%	68%
Poor	33%	21%
Unknown	5%	11%
Number of multiple infarcts	7	8
Age at first infarct	40	41
Months postinfarct #1	64 3	63 8
Months on study	53 8	54 9
Serum cholesterol	256	238
C/P ratio	0 90	0 87
Height	67 inches	68 inches
Weight	162 lb	166 lb
Number of lost patients	6	21
Total number of deaths and (%)	10 (27 0%)	8 (14 3%)
Number of non CVR deaths and (%)	0	3 (5 4 %)
Number of CVR deaths during first 2 months and (%)	0	1 (1 8 %)
Number of coronary CVR and unknown deaths after first 2 months ("valid deaths) and (%)	10 (27 0%)	4 (7 7 %)

the several criteria utilized The only differences are in lost patients and mortality The placebo group experienced a patient drop out rate of 15% in 3½ years the estrogen group 38% [3] This sizable difference is undoubtedly a consequence of the estrogen induced side effects gynecomastia decrease in libido and potency [4] In contrast to the vir



	[illegible] %	[illegible] % G	P	U	No. with Mult. [illegible]	No. with [illegible] Hypertension	Age Infarct	Serum Cholesterol + / [illegible]	Height Weig	No. started on low dosage [illegible]	Total No. of [illegible] d	Total No. of Deaths and %	No. of non-CVR deaths %	No. of CVR death during 1st 2 mo. %	No. of Valid death %
PLAC 80 3½	58	3	30		7	2		56 0 60		0		2	0	0	3
ROGE 3½ YRS	6	64		10	10	2 4	3	2 88		5	38	7	3	2	3 5

FIG 8 Survival during first 3½ years of study (data as of March 1958) The data in the upper right hand corner are on the 3 groups—reading from above down—all estrogen patients except non cardiovascular renal deaths (58 patients) all estrogen patients (61) all placebo patients (40) As the tabulation at the bottom of the graph indicates there were no non CVR deaths in the placebo patients there were 3 non CVR deaths in the estrogen patients Survival curves are plotted based on all deaths CVR deaths only and CVR deaths after the first 2 months of treatment

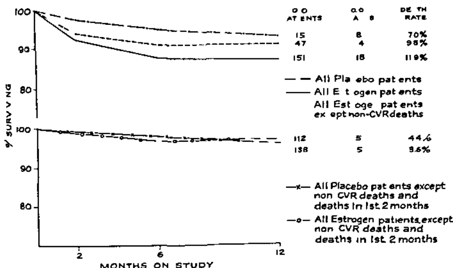
tually universal occurrence of these side effects in estrogen treated patients none of them complained of toxic effects e g anorexia nausea vomiting diarrhea malaise This freedom from toxic effects with large doses of Premarin is different from the experiences with ethinyl estradiol

The findings with respect to patient losses nonfatal recurrences, and

[3] Based on tracing a high per cent of these lost patients no deaths are recorded among these 6 and 23 cases respectively

[4] No feminizing effects on voice or on fat distribution were noted

deaths for all 276 patients are summarized in Tables V and VI The mean duration since first infarct was 47 3 and 49 0 months for placebo and estrogen groups respectively the mean duration under study 35 9 and 37 7 months respectively Recurrence rate of nonfatal myocardial infarction over all death rate and death rate due to coronary and



	Series %	% G		U	No with Multiple Infarcts	No with Diabetes an Hypertension	Age at infarct	Serum Cholesterol / ratio	Height Weight	No. ... on low dosage ... mg	Total No of lost patients /	Total No of Deaths and %	No of non-CVR deaths %	No of CVR deat during 1st 2mo. R %	No of ... de the %
PLAC YR	5	43	32	5				56 0	68 66			8	0		
STROG YR			36			10				96		8	9		

Fig 9 Survival during first year of study (data as of March 1958) The data in the upper right hand corner are on the 3 groups—reading from above down—all placebo patients (115) all estrogen patients except non cardiovascular renal deaths (147) all estrogen patients (151) As the tabulation at the bottom of the graph indicates there were no non CVR deaths in the placebo patients there were 4 non CVR deaths in the estrogen patients Survival curves are plotted based on all deaths CVR deaths only and CVR deaths after the first 2 months of treatment

cardiovascular renal causes were all essentially similar in the estrogen and placebo groups and subgroups These over all data combining experiences on patients studied for both relatively brief and relatively prolonged periods of time do not indicate a positive therapeutic effect of estrogen (cf also Fig 9)

One other aspect of these data needs emphasis Before June 1954 i e before the initial dosage became 10 mg of Premarin coronary and

cardiovascular renal deaths during the first 2 months of treatment were rare In fact only 1 case occurred among 56 patients (Tables III and IV Fig 8) In contrast such deaths became disturbingly frequent—8 cases among 101 patients a rate of 7.9% compared with 2.5% for the placebo group—after the institution of an initial Premarin dosage of 10 mg (Tables VI and VII Fig 9) This difference in the frequency of this phenomenon is sizable enough to call attention to it and sound a warning Its mechanism is not clear Possibly it is a chance phenomenon, although that is unlikely Perhaps it is related to known effects of es-

TABLE V

LOSSES AND NONFATAL RECURRENCES—(DATA AS OF OCTOBER 1957)

Group[a]	N	Losses		Nonfatal recurrences[b]	
All placebo	119	23	19.3%	17/12	14.3%
All estrogen	157	43	27.4%	20/18	12.6%
Series—placebo	68	14	20.6%	5/3	7.4%
Series—estrogen	97	21	21.6%	12/10	12.4%
S Pl G	56	13	23.2%	3/2	5.4%
S E G	66	14	21.2%	7/5	10.6%
OS Pl	51	9	17.6%	12/9	23.5%
OS E	60	22	36.7%	8/8	13.3%
All G-Pl	75	15	20.0%	5/4	6.7%
All G-E	91	19	20.9%	9/7	9.9%
All P Pl	38	5	13.2%	11/7	28.9%
All P E	56	19	33.9%	10/10	17.9%

[a] S Pl G is the series placebo good risk group S E G is the series estrogen good risk group OS Pl is the off series placebo group OS E is the off series estrogen group All G-Pl is the all good risk placebo group All G-E is the all good risk estrogen group All P Pl is the all poor risk placebo group All P E is the all poor risk estrogen group

[b] In the column on nonfatal recurrences the 17/12 value and others similar to it represent 17 nonfatal recurrences of myocardial infarction in 12 patients i e some patients had multiple nonfatal recurrences The per cent is based on the total number of recurrences e g 17/119 = 14.3%

trogens on clotting phenomena (demonstrated with large intravenous doses) or on water electrolyte balance In any case this experience has led us to recommend—to those physicians planning to use estrogens—that a start be made with lower dosages with a gradual build up to higher ranges if so desired

This statement should not be misconstrued Our group is not advising estrogen therapy for coronary disease We are merely relaying our experience and based on it, urging caution upon those who are electing to use estrogens at this juncture

Our assessment of the data accumulated to date is that they still leave unsettled the problem of the possible efficacy of estrogens in the long

TABLE VI
DEATH DATA—ALL PATIENTS AS OF MARCH 1 1958

Group	N[a]	Total deaths and %	Non CVR deaths and %	CVR deaths in 1st 2 months of treatment and %	Coronary CVR and unknown deaths after 1st 2 months (valid deaths) and %
All	276	40(14.5%)	4(1.4%)	12(4.3%)	24(9.2%)
All placebo	119	15(12.6%)	0(0 %)	3(2.5%)	12(10.3%)
All estrogen	157	25(15.9%)	4(2.5%)	9(5.7%)	12(8.3%)
Series placebo	68	8(11.8%)	0(0 %)	3(4.4%)	5(7.7%)
Series estrogen	97	18(18.6%)	2(2.1%)	7(7.2%)	9(10.2%)
Series placebo good risk	56	5(8.9%)	0(0 %)	1(1.8%)	4(7.3%)
Series estrogen good risk	66	10(15.2%)	[illegible](3.0%)	4(6.1%)	4(6.7%)
Series placebo poor risk	9	2(22.2%)	0(0 %)	1(11.1%)	1(12.5%)
Series estrogen poor risk	24	6(25.0%)	0(0 %)	3(12.5%)	3(14.3%)
Off series placebo	51	7(13.7%)	0(0 %)	0(0 %)	7(13.7%)
Off series estrogen	60	7(11.7%)	2(3.3%)	2(3.3%)	3(5.4%)
Off series placebo good risk	19	0(0 %)	0(0 %)	0(0 %)	0(0 %)
Off series estrogen good risk	25	1(4.0%)	1(4.0%)	0(0 %)	0(0 %)
Off series placebo poor risk	29	7(24.1%)	0(0 %)	0(0 %)	7(24.1%)
Off series estrogen poor risk	32	5(15.6%)	1(3.1%)	1(3.1%)	3(10.0%)
All good risk placebo	75	5(6.7%)	0(0 %)	1(1.3%)	4(5.4%)
All good risk estrogen	91	11(12.1%)	3(3.3%)	4(4.4%)	4(4.8%)
All poor risk placebo	38	9(23.7%)	0(0 %)	1(2.6%)	8(21.6%)
All poor risk estrogen	56	11(19.6%)	1(1.8%)	4(7.1%)	6(11.8%)

[a] N is the number of patients

Non CVR deaths and deaths in the first two months subtracted from the denominator as well as the numerator in calculating per cent "valid" deaths

TABLE VII
ALL DEATHS IN THE FIRST TWO MONTHS ON STUDY

Group	Risk	*Patient* number	*Months post* infarct	*Time on* study	Cause of death	Drug dosage (mg)
Series placebo	Good	15 0010	2	10 days	Suspected infarct	—
Series placebo	Poor	12 0208	3	1 month	Proven infarct	—
Series placebo	Unknown	14 0006	—	1 day	Unknown	—
Series estrogen	*Good*	*15 0003*	*4*	*1 month*	*Proven infarct*	*10 0*
Series estrogen	Good	15 0011	3	1 month	Suspected infarct	10 0
Series estrogen	Good	14 0017	4	< 1 month	Suspected infarct	10 0
Series estrogen	Good	12 0219	1	2 months	Suspected infarct	10 0
Series estrogen	Good	11 7625	1	10 days	Alcohol antabuse reaction	1 25
Series estrogen	Poor	11 7632	7	2 months	Unknown	1 25 T
Series estrogen	Poor	14 0013	2	1 month	Proven infarct	10 0
Series estrogen	Poor	12 0207	4	*2 months*	*Proven infarct*	*10 0*
Off series estrogen	Poor	13 0601	79	< 1 month	Proven infarct	10 0
Off series estrogen	Poor	12 0169	9	2 months	Suicide	1 25
Off series estrogen	Unknown	13 0463	75	2 months	Unknown	10 0

term treatment of coronary heart disease The survival curves for the estrogen treated group on therapy for 3½ years are suggestive of a favorable therapeutic effect However they are not conclusive since the numbers of both patients and deaths are too small The over all data on all patients are not encouraging However the period of follow up is still too brief It is anticipated that a clear cut answer should be forthcoming by 1960 In the interim we wish to emphasize that no definitive evidence is available warranting the incorporation of estrogens in the general therapeutic armamentarium for clinical coronary disease

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References

1 Barr D P Russ E M and Eder H A *Am J Med* **11** 480 (1951)
2 Eilert M L *Am Heart J* **38** 472 (1949)
3 Furman R H Howard R P and Imagawa R *Circulation* **14** 490 (1956)
4 Gertler M M and White P D Coronary Heart Disease in Young Adults Harvard Univ Press Cambridge Massachusetts 1954
5 Higginson J and Pepler W J *J Clin Invest* **33** 1366 (1954)
6 Katz L N Stamler J and Pick R Nutrition and Atherosclerosis Current Status of the Problem (A Monograph) Lea and Febiger Philadelphia Pennsylvania (in press)
7 Mann G V Munos J A and Scrimshaw N S *Am J Med* **19** 25 (1955)
8 Marmorston J Lewis J J Bernstein J D Sobel H Kuzma O Alexander R Magidson O and Moore F J *Geriatrics* **12** 297 (1957)
9 *Oliver M F and Boyd G S Am Heart J 47 348 (1954)*
10 Page I H Lewis L A and Gilbert J *Circulation* **13** 675 (1956)
11 Rivin A U and Dimitroff S P *Circulation* **9** 533 (1954)
12 Russ E M Eder H A and Barr D P *Am J Med* **11** 468 (1951)
13 Scrimshaw N S Trulson M Tejada C Hegsted D M and Stare F J *Circulation* **15** 805 (1957)
14 Stamler J *J Natl Med Assoc* **50** 161 (1958)
15 Stamler J *J Am Dietet Assoc* **34** 701 814 929 1053 1060 (1958)
16 Stamler J Pick R and Katz L N *Ann N Y Acad Sci* **64** 596 (1956)
17 Walker A R P and Arvidsson U B *J Clin Invest* **33** 1358 (1954)
18 Wuest J H Dry T J and Edwards J E *Circulation* **7** 801 (1953)

CHAPTER 31

Studies with Manvene[1]

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Estrogen administration to middle aged men with coronary heart disease has been complicated by undesirable estrogenic side effects which sharply limit its application to large numbers of male patients In an attempt to avoid these distressing symptoms synthetic analogs of both steroidal and nonsteroidal estrogens have been used in attempts to find a substance that retains lipid shifting potency and yet lacks estrogenicity One of these estrogen analogs synthesized in the laboratories of G D Searle and Co 3 methoxy 16α methyl 1 3 5(10) estratriene 16β 17β diol (Manvene) showed lipid shifting and antiatherogenic effects in the chick but only slight estrogenic activity when tested in the mouse uterine growth or rat vaginal smear assays (1) The compound was free of serious side effects in toxicity studies (1) thus allowing its use in clinical trials (2) The outcome of a 6 month period of therapy with oral Manvene is the basis of this report

MATERIAL AND METHODS

Clinical Material

Forty nine middle aged males who had recovered from myocardial infarction were selected for study Twenty nine patients were divided into three groups and placed on 2 5 5 0 or 10 0 mg of Manvene daily for 6 months For comparison 20 patients were placed on 10 0 mg daily of oral, mixed conjugated equine estrogens (Premarin)

Estrogenic side effects in both the Premarin and Manvene groups were graded according to the schema of Table I

Biochemical Methods

Total serum cholesterol lipid phosphorus and cholesterol content of the α and β lipoproteins after preparative ultracentrifugal separation were determined by the standard methods of this laboratory (3 4)

RESULTS

At 1 month of therapy both 5 and 10 mg dosage levels of Manvene and 10 mg of Premarin had shown similar lowering effects on the C/P

[1] This work was supported by grants from G D Searle and Co and the Worcester District Chapter Massachusetts Heart Association

Cholesterol Phospholipid

ratio But beyond 1 month the Premarin group showed a sustained decrease nearly double that of the 5 and 10 mg Manvene groups There was a smaller decrease in the subjects treated with 2 5 mg of Manvene

Comparable effects were seen in the β /α lipoprotein cholesterol ratios during therapy Again the Premarin treated group showed a greater response than did the Manvene treated groups

TABLE I
Scoring of Estrogenic Side Effects

Side effects	2 months	6 months
Breast tenderness		
Slight	1	1
Moderate to severe	2	2
Breast hypertrophy		
Slight	2	2
Moderate to severe	4	4
Depression of sex functions		
Slight to moderate	1	1
Complete	2	2
Maximum possible score	8	8
	Total 16	

The graded side effects derived according to Table I are shown in Fig 1 for all groups The mean side effects score of the Premarin group was 10 8 range 8–14 The mean score of the 2 5 mg Manvene group was 2 4 and that of the combined 5 and 10 mg group 6 6 Analysis of the individual responses to Manvene was carried out by constructing a scattergram to determine whether this substance is in fact a non estrogenic lipid shifting agent

In the upper portion of Fig 2 the individual responses of the Premarin group are shown in a scattergram using as a measure of lipid shifting effects the sum of the percentage decreases of the β /α lipoprotein cholesterol ratios at 2 and 6 months of therapy plotted against the sum of the estrogenic side effects scores evaluated at the same time In all but one patient there was excellent serum lipid response accompanied by significant side effects

The corresponding scattergram of the Manvene treated groups is shown in the lower portion of Fig 2 In contrast to the Premarin group only 9 Manvene treated patients showed both extensive serum lipid changes and severe side effects Twelve patients had little or no side effects accompanied by minimal serum lipid changes There were 8 patients showing a dissociation 5 of whom were considered to be favor

able because of significant serum lipid response with slight side effects Analysis of data from a preliminary study of higher Manvene dosages (2) showed that 50 mg of this drug produced serum lipid changes very similar to those obtained with 10 mg of Premarin This Premarin/Manvene "potency ratio" of 5 is similar to that of estrone/Manvene in terms of the C/P ratio lowering effect in cholesterol fed chicks also found to be about 5 (1)

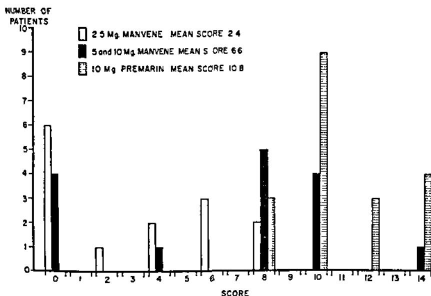


FIG 1 Side effects scores of patients treated with Premarin or various dosages of Manvene Scores are sums of side effects noted at 2 and 6 months of therapy

Comparative studies of different estrogens using the immature mouse uterus test revealed that Manvene had only 0 3 to 3% of the estrogenic activity of Premarin (1) This relative lack of estrogenicity has not been borne out by the results in men since the side effects scores of patients on 5 or 10 mg of Manvene daily were often similar to those of the 10 mg Premarin group The high incidence of estrogenic side effects in the 50-mg Manvene group prevented the continuation of this dosage beyond 2 months

The conventional biologic tests for estrogenic activity have failed to predict the estrogenic potency ratio of Premarin/Manvene in men It is suggested that the response of the vaginal epithelium of healthy post menopausal women might be used for the crude estimation of estrogenicity of new lipid shifting compounds related to the steroidal

estrogens before these substances are administered to men. A similar lack of correspondence between estrogenic potency in human beings and in experimental animals has been reported by Brown and Bradbury (5).

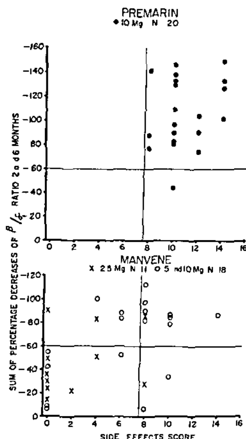


Fig 2 Scattergrams comparing individual lipid shifting and estrogenic side effects of Premarin and Manvene

Summary

Estrogenic side effects and serum lipid changes were studied in middle aged men with coronary heart disease during a 6 month therapy period with different doses of Manvene, *a new estrogen analog*. Although significant lipid shifting effects were noted at 5 and 10 mg daily these were not comparable with those of 10 mg of Premarin. Analysis of scattergrams of individual estrogenic and lipoprotein responses of both Manvene and Premarin treated patients revealed a favorable dissociation of these effects in 5 of the 29 men in the Manvene groups

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REFERENCES

1 Cook D L and Edgren R A Personal communication (1957)
2 Robinson R W Cohen W D and Higano N *Circulation* **14** 489 (1956)
3 Robinson R W Cohen W D and Higano N *Ann Internal Med* **48** 95 (1958)
4 Robinson R W Higano N Cohen W D Sniffen R C and Sherer J W Jr *Circulation* **14** 365 (1956)
5 Brown W E and Bradbury J T *J Clin Endocrinol* **9** 725 (1949)

CHAPTER 32

Hormonal Treatment in Arteriosclerotic Disease[1]

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I would like to outline briefly the experiment as we will report it We started to study this disease about 4 years ago in menopausal female subjects so that we did the hormonal and lipid excretion levels in meno pausal females About 18 months ago we started to study male subjects and in this group we studied the lipid and hormonal excretion levels as well We have some results in the menopausal females with estrogen therapy which we would like to report here We would like to tell you a little bit about our approach as far as the male experiment is con cerned Dr Adlersberg suggested that after each survey of the litera ture all the various population groups are so different and so we thought that we should take a look at the death rates in our hospital Briefly I will show you also the death rates in our hospital other than in those patients who are involved in our particular experiment This experiment is being conducted in two hospitals There is a clinic in the Cedars of Lebanon Hospital where the large majority of the patients are Jewish, and we have several clinics in the Los Angeles County Hospital Since the majority of patients come from the County Hospital we sur veyed the death rate in the females These patients were classified as to age at the time of their first infarct—the age was approximately 66 and among those who died 69 years of age (Tables I and II) There were 400 females who were surveyed by our Chief Clinician Dr Oscar Magidson The Negro patient in our hospital dies at approximately age 60 (Table III) This is the mortality in the first 6 weeks and the over all mortality in females for this year was 56%—for the first infarct a little over 41% for patients with subsequent infarct—58% and the Negro as recorded here has a very high mortality of 68% These figures are so different from those reported from other hospitals that we thought you ought to see them (Table IV) This is the mortality by age group and for the first myocardial infarction to 6 weeks Under 65 years of

[1] The investigations described in this paper were aided by grants from the United States Public Health Service (H 2198 2701) and the Lasker Foundation

[2] We would like to acknowledge with grateful thanks the work of Dr Harry Sobel who in the early phases of this project, contributed many important ideas in the planning of the experiments and did some of the early lipid and hormonal excretion levels on some of the patients herein reported The majority of the lipid studies were made under the direction of Dr John W Mehl

age the mortality is a little over 26% at 65–75 years of age the mortality rate is over 41% and over 75 years of age 58% Table V shows the very high death rate during the follow up period At the end of 6 weeks 56% of our females are dead and at the end of 3 months 60% and at the end of 15 months 72% but obviously as Table V shows most of the deaths occur during the first 3 months

TABLE I

AGE OF PATIENTS AT TIME OF FIRST MYOCARDIAL INFARCTION

Cases	Age (years)
All cases	66 6
Live patients	66 0
Deaths	69 2
Negro	60 2

TABLE II

ALL FIRST ATTACKS — AGE AT TIME OF FIRST INFARCT

Age	Number	Per cent	Total per cent
40–45	3	1 9	
46–50	2	1 3	
51–55	12	7 7	
56–60	21	13 4	24 3
61–65	23	14 7	
66–70	35	22 4	61 4
71–75	27	17 3	
75–80	24	15 4	
81–85	8	5 1	
86–90	1	0 7	100
66 6	156	100	

TABLE III

MORTALITY DURING THE FIRST SIX WEEKS

Cases	Per cent
All female myocardial infarctions fiscal year 1955–1956	56 2
First myocardial infarction	41 7
Subsequent myocardial infarction	58 0
Mortality Negro patient 1st MI	58 6

Tables VI and Figs 1 to 6 summarize our results in studies of serum cholesterol phospholipids beta lipoproteins and the S_f fractions in various clinical and age groups These findings generally tend to support the much more extensive work already done in this field by others We would like to present certain observations on the lipids which are of considerable interest We begin by demonstrating the well established

correlation between cholesterol and phospholipids in women Table VI simply shows that the serum cholesterols in infarcts are higher than in the well controls and distinctly depressed in the sick controls (Table VII) The phospholipids are higher in the infarcts than in the well and again distinctly depressed in the sick controls (Table VIII) The C/P ratios are about the same in these clinical groups Table IX

TABLE IV
MORTALITY BY AGE GROUPS FOR FIRST MYOCARDIAL INFARCTION TO SIX WEEKS

Age (years)	Number of cases	Per cent mortality
Under 65	42	26 6
65–75	48	41 6
Over 75	36	58 4

TABLE V
MORTALITY (ALL CAUSES) TO END OF 15 MONTHS

Time	Number dead	Per cent dead
0–6 weeks	184	56 2
7 weeks–3 months	196	60 0
4–6 months	209	64 5
7–9 months	220	67 5
10–12 months	230	71 0
13–15 months	230	72 0

TABLE VI
SERUM CHOLESTEROL IN POSTMENOPAUSAL WOMEN

	Well controls WC		Sick controls SC		Myocardial infarcts MI		p values		
Age	n	x^b	n	x^b	n	x^b	WC SC	WC MI	SC MI
To 55	14	293	8	212	12	282	0 01	—	0 05
56–65	18	273	5	252	21	283	—	—	—
66 or more	11	262	5	264	36	286	—	—	—
All ages	43	277	18	236	69	285	0 05	—	0 02

Number of cases
[b] Geometric mean in mg %

shows the serum cholesterol in men higher in the infarct than in the well and lower in the sick controls than in either of the other two groups Table X shows that the serum phospholipids in men are higher in the infarcts than in the well and still lower in the sick controls probably due to inanition These sick controls are patients who have a variety of diseases other than cardiovascular Table XI shows the C/P

ratios in man and they tend to be higher in the infarcts than in the well controls and again they are lower in the sick controls Table XII shows that the percentage beta serum lipoprotein in men is higher in the MIs than in the well controls and about as low in the sick as in the well controls The correlation of cholesterol and phospholipids is positive in women (Table XIII) and also in men (Table XIV)

TABLE VII

SERUM PHOSPHOLIPIDS IN POSTMENOPAUSAL WOMEN

	Well controls WC		Sick controls SC		Myocardial infarcts MI		p values		
Age	n[a]	x[b]	n[a]	x[b]	n[a]	x[b]	WC SC	WC MI	SC-MI
To 55	14	273	8	217	12	264	0 01	—	0 02
56–65	18	264	5	236	21	287	—	—	—
66 or more	11	282	5	264	36	281	—	—	—
All ages	43	272	18	235	69	280	0 05	—	0 01

[a] Number of cases

[b] Geometric mean in mg %

TABLE VIII

CHOLESTEROL/PHOSPHOLIPID RATIO IN POSTMENOPAUSAL WOMEN

	Well controls WC		Sick controls SC		Myocardial infarcts MI		p values		
Age	n[a]	x[b]	n	x[b]	n[a]	x[b]	WC SC	WC MI	SC MI
To 55	14	1 07	8	0 97	12	1 07	—	—	—
56–65	18	1 03	5	1 07	21	0 99	—	—	—
66 or more	11	0 93	5	1 00	36	1 02	—	0 02	—
All ages	43	1 02	18	1 01	69	1 02	—	—	—

[a] Number of cases

[b] Geometric mean C/P ratio

TABLE IX

SERUM CHOLESTEROL IN MEN

	Well controls WC		Sick controls SC		Myocardial infarcts MI		p values		
Age	n[a]	x[b]	n[a]	x[b]	n[a]	$\bar{x}$[b]	WC SC	WC-MI	SC MI
To 39	18	233	9	192	7	254	0 05	—	0 02
40–59	22	239	29	184	35	256	0 001	—	0 001
60 or more	10	199	31	184	37	242	—	0 01	0 001
All ages	50	228	69	185	79	249	0 001	0 02	0 001

[a] Number of cases

[b] Geometric mean in mg %

We then studied the urinary excretion level of estrogens ketosteroids and corticoids in postmenopausal women and in male subjects with myocardial infarction The preliminary results have been published elsewhere (*Geriatrics* 12 297 1957)

Using small doses of estrogen given to male and female subjects we have studied the lipid changes observed under treatment others have

TABLE X
SERUM PHOSPHOLIPIDS IN MEN

	Well controls WC		Sick controls SC		Myocardial infarcts MI		p values		
Age	n[a]	$\bar{x}$[b]	n[a]	$\bar{x}$[b]	n[a]	$\bar{x}$[b]	WC SC	WC-MI	SC-MI
To 39	18	231	9	191	7	261	0.01	—	0.001
40–59	22	245	29	207	35	241	0.01	—	0.01
60 or more	10	217	31	199	37	237	—	—	0.01
All ages	50	234	69	201	79	241	0.001	—	0.01

[a] Number of cases
[b] Geometric mean in mg %

TABLE XI
SERUM C P RATIO IN MEN

	Well controls WC		Sick controls SC		Myocardial infarcts MI		p values		
Age	n	$\bar{x}$[b]	n	$\bar{x}$[b]	n	$\bar{x}$[b]	WC-SC	WC-MI	SC-MI
To 39	18	1.01	9	1.01	7	0.97	—	—	—
40–59	22	0.98	29	0.89	35	1.06	—	0.05	0.001
60 or more	10	0.92	31	0.93	37	1.02	—	0.05	—
All ages	50	0.98	69	0.92	79	1.04	—	0.05	0.001

[a] Number of cases
[b] Geometric mean C/P ratio

TABLE XII
SERUM LIPOPROTEINS IN MEN

	Well controls WC		Sick controls SC		Myocardial infarcts MI		p values		
Age	n	$\bar{x}$[b]	n	$\bar{x}$[b]	n	$\bar{x}$[b]	WC-SC	WC-MI	SC-MI
To 39	18	77	9	74	7	85	—	0.02	0.01
40–59	21	77	24	76	34	81	—	—	0.01
60 or more	9	74	31	76	36	80	—	0.01	0.01
All ages	48	77	64	76	77	81	—	0.001	0.001

Number of cases
[b] Mean per cent β lipoprotein

noted that estrogen lowers cholesterol and the C/P ratio but they have generally used much larger doses Figure 1 shows the pre and post treatment levels of cholesterol phospholipids and C/P ratio in post menopausal women receiving only 10 μg of ethinyl estradiol daily for periods of up to 28 months The (A) curve represents findings during the first 2 months the (B) during the 3 to 6 months and (C) 7 to 28 months The fall in cholesterol is greatest in those whose initial level is high In the phospholipids the proportional increase under treatment

TABLE XIII

CORRELATION OF CHOLESTEROL WITH PHOSPHOLIPIDS IN WOMEN

	Well controls		Sick controls		All controls		Myocardial infarcts	
Age	n[a]	r[b]	n[a]	r[b]	n[a]	r[b]	n[a]	r[b]
To 55	12	+0 58	6	+0 92	18	+0 80	10	+0 60
56–65	13	+0 93	4	+0 87	17	+0 88	15	+0 86
66 or more	6	+0 94	5	+0 97	11	+0 91	20	+0 95
All ages	31	+0 82	15	+0 87	46	+0 85	45	+0 85

[a] Number of cases
[b] Correlation coefficient

TABLE XIV

CORRELATION OF CHOLESTEROL WITH PHOSPHOLIPIDS IN MEN

	Well controls		Sick controls		Myocardial infarcts		All cases	
Age	n[a]	r[b]	n[a]	r[b]	n[a]	r[b]	n[a]	r[b]
To 39	15	+0 85	9	+0 19	7	+0 83	31	+0 77
40–59	18	+0 52	28	+0 72	33	+0 47	79	+0 70
60 or more	5	+0 82	29	+0 69	32	+0 63	66	+0 72
All ages	38	+0 73	66	+0 66	72	+0 59	176	+0 71

[a] Number of cases
[b] Correlation coefficient

was greatest and earliest in those patients whose initial level was low In the C/P ratio those with a low ratio before treatment stayed the same while those with a high ratio fell under therapy With these low doses the change was progressive over 6 months with no tendency to escape during longer periods of observation (up to 44 months)

Figure 2 shows the changes in cholesterol in men under ethinyl estradiol The dosages used prior to the lipid levels reported here ranged from 10 to 200 μg the median being 100 μg Figure 2 shows that the changes in men are both quicker and more marked than in women with much smaller doses If the pretreatment level of cholesterol was high it fell under therapy if low it tended to rise under therapy The maxi

mum changes are found after 3 months The phospholipids (Fig 3) show the same thing but the maximum change is not observed until after 6 months Those with high phospholipids fall under therapy those with low phospholipids tended to rise Estrogens therefore tended to bring both cholesterol and phospholipids to normal" levels regardless of their pretreatment levels The C/P ratio (Fig 4) under estrogen therapy tends to reach a value of somewhat less than 0 9 no matter what it was before treatment These effects of estrogen are consistent with the view that it may have a homeostatic effect This study shows that small doses have a marked "normalizing" effect on the lipids Cholesterol and C/P ratio rose in untreated male MI s The changes of the per cent beta

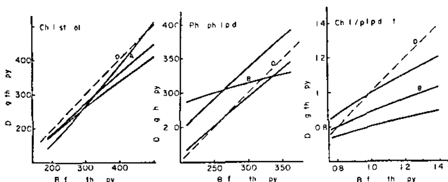


Fig 1 Effect of small doses of ethinyl estradiol on the blood lipids in post menopausal women with myocardial infarction

lipoprotein under ethinyl estradiol are shown in Fig 5 Note that the per cent of beta lipoprotein under treatment is brought to low normal levels very quickly The comparison of the rate of change in the various lipids and lipoproteins indicates that the per cent beta lipoprotein changes occur earlier and more markedly than do the changes in cholesterol but this is not shown in Fig 5 as such

In Fig 6 we have studied the effects of estrogen on the 0–12 S_f fraction There is little change under 3 months therapy treatment for more than 3 months tends to reduce the amount of this fraction in those patients who had high levels initially The findings in the 12–20 S_f fraction (Fig 7) are of a similar nature to those shown in the previous figure—no change in the first 3 months with a lowering of high values with treatment longer than 3 months In the case of the 20–100 S_f fractions (Fig 8) we find that even short term therapy reduces the level if initially elevated With prolonged therapy the amount of this fraction tends to reach essentially normal levels no matter what the pretreatment

value may have been Figure 9 shows the S_f 100–400 on the sa therapy The findings are essentially the same as those in the S_f 20-1

We would like to mention our current clinical trials of treatment women with small doses of ethinyl estradiol The clinical trial in po

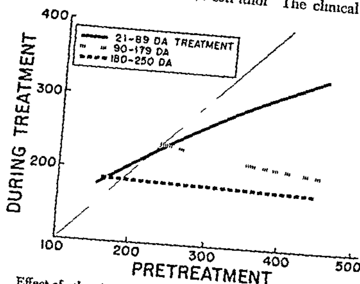


Fig 2 Effect of ethinyl estradiol on cholesterol in male myocardial infarction

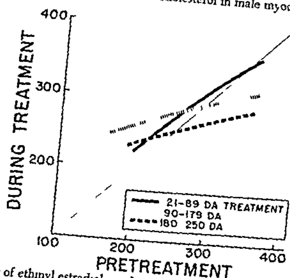


Fig 3 Effect of ethinyl estradiol on phospholipids in male myocardial infarction

menopausal women was divided into two experiments In the early phase of our work the women were not assigned to treatment or control groups by a randomized method In this experiment postmenopausal women with myocardial infarction whose hormones and blood lipid

levels had been studied were placed on continuous estrogen therapy with small doses of 10 µg of ethinyl estradiol For controls the hospital populations were searched in an effort to find women with myo-

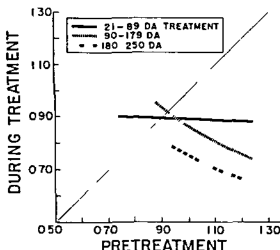


FIG 4 Effect of ethinyl estradiol on cholesterol phospholipid ratio in male myocardial infarction

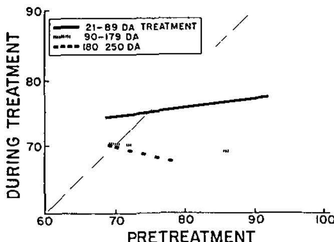


FIG 5 Effect of ethinyl estradiol on per cent beta lipoproteins in male myocardial infarction

cardial infarctions whose chief clinical characteristics matched those of our patients under treatment Their age range was from 45 to 80 The dose was the same in all patients The patients were not included in

this report unless on treatment at least 2 months The treatment patients are now being seen in our clinics every 3 to 4 weeks The control un treated myocardial patients are being seen every 4 weeks and are re ceiving placebos As in the random series certain disease states such

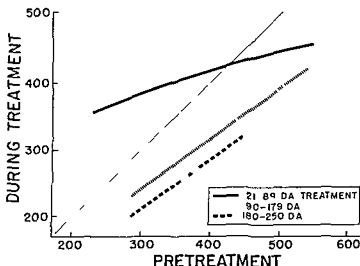


Fig 6 Effect of ethinyl estradiol on S_f^0 0–12 fraction in male myocardial infarction

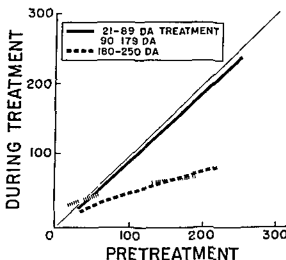


Fig 7 Effect of ethinyl estradiol on S_f 12–20 in male myocardial infarction

as diabetes hypertension hypo or hyperthyroidism nephrosis and xanthomatosis were excluded

The time of infarction was within 2 years in all cases except 1 pair which was 3 years The secondary matching was on time of infarct the

controls having remained alive long enough to have "initiated treatment" after the same interval of time as the treatment patient There are 54 matched pairs or 108 patients in this experiment Of these 54 pairs there were 16 pairs in which the control was the first to die and

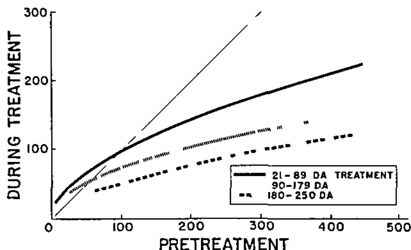


FIG 8 Effect of ethinyl estradiol on S_f 20–100 in male myocardial infarction

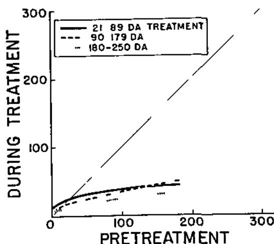


FIG 9 Effect of ethinyl estradiol on S_f 100–400 in male myocardial infarction

there were 6 pairs in which the treated patient was the first to die this difference carrying a *P* value of 0 02

In the second experiment men and women recovering from myocardial infarction at the Los Angeles County and at the Cedars Hospitals were

assigned to treatment in four groups by the statistical office using stratified randomization Most patients were started on approximately 30 μg of ethinyl estradiol and within 2 or 3 clinical visits were placed on 100 μg and in some cases increasing to 200 μg depending on individual tolerance Some patients were placed on Manvene (Searle) or Premarin and today the numbers of patients under treatment are as follows There are 286 male patients of which 137 male patients are on treatment and 76 patients on placebos In the new female randomized clinical trial there are 31 females on treatment and 35 on placebo The untreated patients are receiving placebos in all instances This is a report of 18 months study in the male and females No conclusion can as yet be reached as to the difference in the occurrence of second and third infarcts and in the death rate of the treated versus the nontreated patients

DISCUSSION OF PAPERS BY DRS STAMLER ROBINSON AND MARMORSTON

BOYLE Several questions briefly and a few comments for my good friend Dr Stamler I would like to know from his slides if the follow up was 3 years data You have 3 years data follow up on top but on the 6 year observation have you any comment about the fate of those before 1954 As you remember the smaller dose of estrogen 2 5 mg of Premarin as you have shown earlier without the cholesterol or lipid shifts present I imagine that some of these patients have been put on higher dosage I was wondering if you had any comment on your longer term observations I am fully cognizant of the fact that the longer you run this study the more people drop out because of side effects It is very difficult for us to talk men into becoming women I know that this is a problem that all of us seem to encounter But one specific question you mentioned in closing that 8 out of the 12 deaths were on estrogen I would like to know whether they were in the poor risk group or in the good risk group As you said the individuals dispensing the medication have no idea as to which patient would receive it And I certainly have not had this death rate acutely in the first 2 months if I withheld estrogen from somebody in congestive failure severe angina edema or polycythemia I am wondering if there was any selection made in separating these patients from the severely poor risk versus the good risk patients and if this affected your 8 out of 12 deaths on treatment during the first 2 months Thirdly I would like to know if you have any data more recently as to the response in cholesterol phospholipid ratio lipoprotein changes in the patients with high S_f 20–400 in contrast to those with high S_f 0–12 or 0–20 Dr Marmorston brought this point out showing the very rapid drop in serum lipids or serum lipoproteins above S_f 20–400 which is basically in line with what we are thinking, a triglyceride disease with secondary passive cholesterol defect as opposed to those with high β (or S_f 0–20) which have essential hypercholesterolemia and handle triglycerides normally I bring this out because from my experience in classifying these patients prior to treatment according to their lipoprotein distribution as I presented it in Chicago at the American Heart meeting in November the different types of lipoprotein patterns seen with only the common denominator of elevated serum cholesterol levels that you have very marked and dramatic response of those individuals with the very low density lipoproteins as contrasted to those with the high density β lipoproteins or the S_f 0–20 of Gofman and if you would separate these patients prior to treatment

into 2 groups you would find a very dramatic difference in their response to estrogen both as to dose response level rapidity of response and also the sometimes 5 to 10-fold drop in lipid levels in one group with practically negligible drop in serum β lipoprotein levels in the other So the 2 major different types of β lipoproteins should be differentiated and will clarify results on the degree rapidity and extent of alteration in the serum lipoprotein if these patients are classified prior to onset of therapy

To Dr Robinson I would like to ask if he has any comments to make on some of the other effects be they forward or sideways Estrogen in large doses causes decreased erythrocytes in the hematological changes edema or salt and water retention and urethritis anorexia G I disturbances testicular atrophy and loss of aggressiveness in the male which were not included in his table of grading side effects

STAMLER With respect to Dr Boyle s questions The graph on patients under study for 3½ years dealt overwhelmingly with subjects who started on the lower dosage and were stepwise over a period of months elevated to the 10-mg daily dosage of estrogens The study yielded no meaningful data on episodes during the period of lower dosage because in no case was the period of lower dosage of adequate duration to permit the occurrence of enough episodes to allow evaluation Within a matter of months all these people were put on the 10-mg dosage regimen The evaluation represents an analysis of their survival pattern on that dosage With respect to the question concerning the massive accumulation of lipid lipoprotein data we can only state that it has not yet been subjected to detailed statistical and analytical treatment beyond what has been presented here That remains a task for the future

SAMUELS Dr Robinson will you answer the last question?

ROBINSON We were primarily interested in the feminizing estrogenic side effects of Manvene As such we have used breast tenderness breast hypertrophy and the depression of sex activity as the main things to be scored In any patient who is placed on estrogen therapy of any significant dosage for any prolonged period of time there are these other secondary or associated changes that Dr Boyle mentioned We have not summarized these secondary effects for Manvene but they certainly occur with high dosages of Premarin or stilbestrol when given over a long period of time in other patients that we observed

BOYLE I merely asked this question for one reason in that you brought out the difference in the responses in the gastrointestinal tract between Premarin and ethinyl estradiol where there are qualitative differences in degree of side effects Certain systems might be involved or not involved that is why I was wondering if these other things might also be considered as side effects to see if other qualitative differences such as headaches peripheral edema urethritis occur This might show some qualitative differences in side or really forward effects of estrogen

ROBINSON It is very difficult for me to answer at this time I would be glad to see if we can re-evaluate our data in the light of what you have said

DRILL *I enjoyed Dr Robinson s paper and was gratified to know that his esti*mate of the lipid potency of SC-6924 did agree with ours Dr Robinson also found the clinical doses to be estrogenic in man This result agrees with the experimental findings Although we speak of the steroids with 1% of the estrogenicity of estrone as having a weak estrogenic effect nevertheless they can be estrogenic in man if dosage is high enough As calculated in an accompanying paper 10 mg of SC 6924 administered orally each day should be estrogenic in man I think there is a fairly close agreement between the experimental animal and man in this regard

ROBINSON Manvene was definitely estrogenic in men As we decreased the daily dose from 50 to 20 10 5 2 5 mg of Manvene we observed progressively less lipid and estrogenic effects There was a dissociation in only 5 patients who had good lipid changes and minimal estrogenic side effects Those were the patients who were most encouraging to me

SAMUELS Were these patients tried with other estrogens to be sure that they could respond Dr Robinson?

ROBINSON This same group of patients had been on estrogen treatment alternated with periods of observation on placebo tablets with 4 compounds over a period of about 2 years

SAMUELS It seems when the response of breast tissue is being used as a gauge of estrogenic action you have to be sure that in the males there is adequate tissue for response

KATZ I want to make sure that we do not get so concerned about refinements that we miss the point that a pharmaceutical house must do several things to justify clinical use of estrogens should they ever turn out to be useful clinically as they have not been so far In short there must be a really sharp distinction between the antiatherogenic action and the estrogenic action the former must be retained while the latter is absent or minimal In other words what I would like to say is that a clinician wants an estrogen which is atherogenic but does not decrease libido does not cause breast enlargement or have other side effects Its beneficial action must be judged by a significant increase in the life of patients who have had clinical manifestations of coronary disease Lest Dr Stamler forget to repeat it I want to point out that when we picked 10 mg of Premarin in our study we did so to be sure that we would get an effect Our studies must not be considered as defining the minimum dose of Premarin that might have an effect

STAMLER The point Dr Katz made is extremely important It merits emphasis especially since Dr Drill s remarks touched on it As one of the graphs in our formal presentation showed for all treated patients as a group an estrogenic effect on lipids and lipoproteins was obtained with the 4 0-mg dosage of hormone However in a few patients presumably taking that dosage for several weeks these effects were not observed The dosage was therefore increased from 4 0 to 10 0 mg per day in order to get effects in 100% of patients No intermediate dosage between 4 0 and 10 0 mg was studied

WERTHESSEN I would like to congratulate Dr Marmorston s statistical group on their presentation It brought out one point that interested me greatly I would like to find out if Dr Stamler saw it in his work as well The point is as Dr Marmorston put it that ethinyl estradiol tends to *normalize* the lipid levels High values fall low values rise I would like to ask if this was seen in the Chicago study as well

STAMLER We have not yet made a detailed analysis of the response *patterns* of individuals or subgroups based on initial lipid lipoprotein levels Therefore all that can be said at this time on this point is first all patients on estrogen manifested a rise in α lipoprotein to a level in the female range be that normalization if you will—this being of course a debatable question Secondly all tended to develop cholesterol phospholipid ratios below unity Those who manifested C/P ratios above unity prior to therapy almost invariably exhibited a decline to values below unity Those who were below unity to begin with stayed there Whether they went down further cannot be stated at this point since that specific statistical analysis remains to be accomplished

WERTHESSEN I would like to suggest that the two groups might get together

on just this tiny point because Premarin happens to be an excreted estrogen Ethinyl estradiol tries to mimic the secreted estrogen There is a great deal of literature that indicates that the pharmacology of the natural estrogens estradiol estriol estrone estrone sulfate is quite different when you get down into specific details The apparent difference in response brought out here might be a lead to the pharmacology of these substances in man

HOWARD I have two comments First, when Dr Stamler read his report I wondered whether he made a slip of the tongue because I think he said that a combination of androgens and estrogens in man was invariably estrogenic in respect to lipids and lipoproteins It is the experience of Dr Furman s group that we have had to go up to ratios of about 1 1 (e g 20 mg Premarin 25 mg methyltestosterone) before we got an estrogenic effect in this respect Secondly I wanted to endorse Dr Robinson s point of view that before clinical trial with respect to lipoprotein effects estrogens really should be tested in the menopausal or castrate woman *because animal assay does not accurately portray the estrogenic potency in man* The substance Premarin contains equilin and estrone as sodium sulfates Using castrate women and urinary gonadotropin assays it has been my experience that it takes approximately 2 5 mg per day of Premarin to suppress the high gonadotropin titer Estrone sulfate is somewhat weaker and equilin sulfate is actually stronger only 0 6 to 1 2 mg per day being required for gonadotropin suppression

STAMLER That definitely was a slip of the tongue What should have been stated was that in the small group on 1 25 or 2 5 mg estrogen dosage plus 10 mg of methyltestosterone feminizing effects on secondary sex characteristics were observed not on the lipid lipoproteins The latter in fact remained masculine with both combined therapy and estrogen alone at this dosage Thank you for correcting that

One other point Premarin contains chiefly estrone plus estradiol equilin and equilenin In our limited experience with a few women in whom vaginal smears have been done feminization effects were obtained at the 1 25 or 2 5 mg dosage level

GOOD Being a pediatrician this is a little bit out of my line but I would like to ask three questions although they may be entirely irrelevant The association between pulmonary infection and cardiac reserves is well recognized The effect of estrogen on the phagocytosis of particles by the reticuloendothelial system particularly in increasing the uptake of substances such as radioactive chromium and carbon particles is well documented and has received considerable attention in the field of infectious diseases For this reason I would like to ask whether or not the statistics which are presented by Dr Stamler are valid if one considers the possibility that estrogenic therapy may change the incidence of pulmonary infection I wonder if you have statistics whether or not there was any increase or decrease in the incidence of pulmonary infections in your two groups (treated vs placebo) which may have upset the statistical data utilizing total loss of life as the criterion for comparison

The other problem in question is whether or not estrogenic therapy is associated with increase in the incidence of carcinoma of the breast or other such entities particularly in the female

STAMLER We of course have been very intrigued by the known stimulating effects of estrogen on the reticuloendothelial system As Dr Ruth Pick pointed out yesterday this mechanism has been hypothesized as one among several which might be responsible for the definitively demonstrated antiatherogenic effect of estrogens in the coronaries of chicks No data are available to test this hypothesis

With respect to the occurrence of pulmonary infection or other infections as a

cause of death in the two groups let me re-emphasize that noncardiovascular renal deaths were minimal in either group as one would anticipate in a group of men with clinical coronary disease In fact no infectious disease death occurred in either group

Now with respect to the last question concerning breast malignancy this of course is one of the banes of our existence It is a problem that is checked on in all follow up examinations of patients There have been none Moreover clinicians who have vast experience treating prostatic carcinoma with estrogens reassure us that this is in fact not a problem since they have not observed breast carcinoma in males on long term estrogen therapy Our experience with females is minimal May I therefore refer that to Dr Marmorston

MARMORSTON This is a very important question and one which we are very much aware of in the County Hospital where there are two medical schools and a great many clinicians who have now heard about the use of estrogens in cardiac conditions This question comes up all the time Some clinic patients ask these questions— Doctor is there any danger that I may have a breast cancer from this therapy? We have been paying a great deal of attention to this question because we give small doses of estrogen to females with myocardial infarction over a long period of time I have discussed this question with several eminent men in the field of cancer The general impression is that there is absolutely no evidence that the giving of estrogens for menopausal symptoms has increased the incidence of breast cancer Dr Emerson Day of the Strong Clinic told me that they had 23 000 patients a year and he has seen no evidence whatsoever that there is any connection In our own patients there is as yet no greater incidence of breast cancer in the treated patient than in the untreated

ROBINSON I think the longest experiences with estrogen therapy in females have been Dr Fuller Albright s at the Massachusetts General and Dr Gordan s in San Francisco treating osteoporosis They produced menstrual periods every month for as long as 20 years in postmenopausal women In Albright s series reported by Henneman there were 2 patients with ovarian carcinoma one with endometrial carcinoma and one with carcinoma of the cervix in a 20 year period which was interpreted as no greater than anticipated in this period of time and perhaps even lower than the expected incidence I think Dr Gordan s experience is similar Incidentally I have observed no case of carcinoma of the breast or uterus in any female I have treated with estrogen

JACOBS I would like to show some of the results we have obtained from the fractionation of urinary estrogens using the Brown procedure Dr Walker reported earlier his results using the same procedure on the South African white and Bantu I hope I shall quote Dr Walker s figures properly here

TABLE A
URINARY ESTROGENS (µg /24 hr)

	South African (young males)		California males (age 40–55)	
Estrogen	Bantu	White	Well Controls	Myocardial Infarcts
Estradiol	2 5	1 1	2 4	2 6
Estrone	5 5	4 3	3 3	3 3
Estriol	3 5	2 6	4 7	6 3
	11 5	8 0	10 4	12 2

The California males aged 40–55 years were of two classes well controls and patients who suffered a frank myocardial infarction at least 6 months prior to the investigation

Since we found no difference in the urinary estrogen levels as measured by mouse uterine weight response in well controls and patients with myocardial infarctions we looked into the fractionation of the estrogens Bauld has reported that patients with a myocardial infarction handle a test dose of estradiol differently from the way controls do Bauld found that 60% of the recovered dose was estriol in the controls whereas 75% of the recovered dose was estriol in the myocardial infarction patient Of the endogenous estrogens we found 45% of the urinary estrogens was estriol in the controls and 52% was estriol in the male myocardial infarction patients This is not statistically different

In the females we have studied the following values were found

TABLE B
URINARY ESTROGENS (μg /24 hr)

Estrogen	Well controls	Myocardial infarcts
Estradiol	0 6	0 5
Estrone	0 6	0 8
Estriol	2 7	3 9
	3 9	5 2

Here the estriol represents 70% of the urinary estrogens isolated from the well controls and 75% of the estrogens from the patients with myocardial infarction In our studies to date we have not been able to find any difference in the way the two groups of people handle their estrogens

SAMUELS The chairman would say he would seriously doubt the significance of 60% versus 75% in such measurements I also think that sick controls are better than well controls for this type of comparison since sickness itself effects steroid metabolism

FLOREY Dr Samuels ladies and gentlemen I have a feeling there is a slight conference paralysis coming over the meeting and I wouldn t dream of increasing it, except that Dr Samuels very kindly this morning asked me if I would like to say something about what we are doing Now when in such a position I usually shuffle my feet and I said I would think about it and hoped that he would forget during the course of the day that he had ever made such a proposition but he seems to have a good memory and I thought I did not want to be so rude as to say no" so I will inflict a few slides upon you if you can put up with it—pictures not graphs which will perhaps relieve those who would be confused by figures Can I say first of all that this work has little to do with the subject of the conference but I am going to show you a little stained lipid in some cells Now there are in Oxford a number of us working on the arteries I hope Dr Holman will approve of arteries and particularly of a special part of the artery the endothelium Actually I have had an interest in the endothelium for a very long time because I wrote my Ph D thesis on endothelium and looking through it the other day I was rather gratified to see that nobody had solved the problems that I hadn t succeeded in solving 30 odd years ago I will show you some slides which I think perhaps show something new We have I think been able to demonstrate that in lesions in the aortic walls of rabbits fed cholesterol you do find sudanophil material in the endothelial cells overlying the plaques Now this endothelium was actually dissected off with a little glass knife by my colleague Poole This work is being done with Poole and Sanders

In a piece of endothelium taken off the top of one of these experimental plaques you can see (I'll be glad of Dr McMillan's views on this) that in these cells there is a collection of sudanophil material The sudanophil droplets as far as I can see are inside the cell because they form around the nucleus It does seem that lipids will in fact appear in the endothelial cells of cholesterol fed rabbits and that may have some correlation with the work done by Dr McMillan and the late Dr Duff when they put colloidal thorium dioxide into animals fed cholesterol and found that it accumulated in the endothelium over the plaques but not in normal endothelium Now I am going to suggest that this possibly means that endothelium is in some way damaged when the rabbits have a high blood cholesterol It is odd that it is only over the plaques that this phenomenon is seen it is not seen in the rest of the aorta Something seems to happen to the endothelium and we are interested in the physiology of the endothelium Well now I am going to show you what happens to endothelium when it is damaged mechanically Curiously enough we know extraordinarily little about the properties of arterial endothelium We know very little indeed about its physiology We do not know whether it secretes some people suppose it secretes cement substance We have very little knowledge of its capacity to regenerate

We scraped off some endothelium from the rabbit's aorta and we watched in over 100 animals what happened as regards regeneration At 24 hours after operation you can see an area from which the endothelium was scraped There are little dots which we think are very likely to be platelets but you will notice that there is no big thrombus We never saw a big thrombus in these animals but just a few platelets with an odd cell or two sitting among them and at this stage there is a very thin protoplasmic film extending from the endothelium as though it is going to grow over the area which is denuded Five days after operation a remarkable phenomenon begins to appear Giant multinucleated cells are found which are not seen in the normal rabbit aorta You may occasionally see a binucleate one but we have never seen anything like this and I do not think Dr McMillan has either At 4 months there is moderately normal endothelium with large cells at the growing edge Even at 316 days the defect originally 1 5 to 2 5 cm long was still not covered In other words endothelium in the rabbit's aorta after mechanical damage is very slow in covering a relatively slight defect In 5 rabbits killed between 410 and 499 days after operation the endothelium had completely covered the denuded area but even after that period of time which is a fair span in a rabbit's life you still have multinucleated cells You can recognize the damaged area quite well just by seeing this type of pattern Also at 499 days we could see rather large endothelial cells with inflammatory cells underneath them Some of them are monocytes and some of them are polymorphs in other words even at 499 days after a clean mechanical damage you can still have inflammatory cells in the wall of an artery Now I am just putting it to you when you are considering all these hormones and so on that the artery is a rather peculiar organ I do not know of any other situation that with such a relatively trivial injury takes so long to heal and it may not be without significance in man because a German pathologist named Sinapius (I doubt if he is known in this country he is only known to me and a few others in England) published work in which he followed the incidence of multinucleated cells in the human aorta by methods similar to those which we used Now in children up to 3 years old there are no multinucleated cells but as time passes you get an increasing number of multinucleated cells which are very similar to those which we found in the rabbit Sinapius has not been able to correlate them with atheroma but they do occur around atheromatous plaques and Dr McMillan and his colleagues have in fact also illustrated these multinucleated cells

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